CERTIFICATE OF MAILING
37 C.F.R §1.8

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10/31/05
Date

Name: Michael R. Krawzenek

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Title:	METHODS AND COMPOSITIONS FOR DELIVERY AND RETENTION OF ACTIVE AGENTS TO LYMPH NODES
Appl. No.:	10/044,650
Applicant:	Goins *et al.*
Filed:	January 11, 2002
TC/A.U.:	1632
Examiner:	D. Nguyen
Docket No.:	UTSK:343US
Customer No.:	32425
Confirmation No.	9390

DECLARATION OF BETH A. GOINS, Ph.D.

I declare:

1. I am a U.S. citizen residing at 13202 Hill Forest Street, San Antonio, Texas, 78230. I am an Associate Professor in the Radiology Department at the University of Texas Health Science Center at San Antonio. I have extensive experience in the field of drug delivery, and more particularly, with the use of liposome-encapsulated drug delivery vehicles. References containing examples of my work are included in my *Curriculum Vitae* which is attached at Appendix 1.

2. I have reviewed the Office Action dated June 29, 2005, the specification, and the pending claims for the above referenced application.

3. I understand that the Examiner has rejected claims 1-19, 29-30, 32-34, and 36-40 of the application on the grounds that they are not enabled by the specification. Specifically, I understand that the Examiner contends that the claims should be limited to subcutaneous administration, wherein avidin is injected at the same site or adjacent to the biotin-liposome injection site. I disagree with the Examiner's assessment for the reasons described below.

4. Based on my knowledge and experience in the field of drug delivery, a person knowledgeable in drug delivery, by following the disclosure in the specification, would be capable of using subcutaneous and other routes of administrations such as those disclosed at page 14, lines 14-17, of the specification to practice the claimed invention without undue experimentation.

5. The specification describes how one would administer a first composition comprising a biotin-liposome complex and a second composition comprising an avidin. For example, page 14, lines 14-17, of the specification provide examples of the different types of administration routes that can be used. Administering the first and second compositions to a subject *via* these exemplary routes is routine in the drug delivery field and would not require undue experimentation.

6. Additionally, the specification provides data showing the delivery and retention of liposomes in one or more targeted lymph nodes when practicing the claimed invention *via* subcutaneous, submucosal, and intraperitoneal administration. Examples 12-15 for instance, are directed towards subcutaneous administration, Example 18 concerns intraperitoneal administration, and Example 19 concerns submucosal administration.

7. Published references also confirm that other routes of administration (*i.e.*, other than subcutaneous) can be used to deliver and retain liposomes in one or more targeted lymph nodes when practicing the claimed invention. These references use biotin-liposome complexes in combination with avidin. The references provide data showing the delivery and retention of the biotin-liposome complexes in one or more targeted lymph nodes *via* intrapleural and intraperitoneal administration:

(i) Phillips *et al.*, *J. of Pharmacology and Experimental Therapeutics*, 303(1):11-16 (2002) (Appendix 2). The Abstract in Phillips *et al.* provides:

> An aliquot (1 ml) of technetium-99m (^{99m}Tc)-biotin-liposomes encapsulating blue dye was injected intraperitoneally in rats. Thirty minutes after administration of the ^{99m}Tc-biotin-liposomes, five rats (experimental) were administered avidin (5 mg) intraperitoneally, whereas the remaining five rats served as controls...Significant ^{99m}Tc activity was detected in blue-stained abdominal nodes (4.7%) and mediastinal nodes (2.3%) from the experimental animals, whereas no blue-stained nodes were detectable in the control animals.

(ii) Medina *et al.*, *J. of Pharmaceutical Sciences*, 93(10):2595-2608 (2004) (Appendix 3). The Abstract in Medina *et al.* provides:

> The objective of this study was to develop a more effective liposome-based method for delivering drugs to mediastinal nodes. Nodal uptake was determined after intrapleural injection of the avidin/biotin-liposome system in normal rats. The effect of injection sequence (avidin injected 2 h before biotin-liposomes and vice versa), volume injected, and administered dose of the agents is described...When avidin was injected before ^{99m}Tc-biotin-liposomes, better mediastinal node targeting (15.7%; $p<0.05$) was achieved than when biotin-liposomes were injected first (8.3%) or when only biotin-liposomes were injected (1.0%).

8. I will now address the Examiner's contention that the specification is enabled only to the extent that avidin is injected at the same site or adjacent to the biotin-liposome injection site, and *vice versa*. A person knowledgeable in drug delivery, by following the disclosure in the specification, would be capable of injecting the avidin containing composition at the same site, an adjacent site, or a non-adjacent site to the biotin-liposome injection site, and *vice versa*, without undue experimentation.

9. For instance, in the subcutaneous administration data of Examples 12-15 of the specification, avidin was injected approximately 2 cm proximal to the biotin-liposome injection site. With respect to the submucosal administration data in Example 19, avidin was injected approximately 5 cm distal to the biotin-liposome injection site. These data show that the avidin and biotin-liposome containing compositions can be injected at non-adjacent sites.

10. Also, I have additional data concerning the administration of the avidin and biotin-liposome containing compositions at different sites (Medina *et al.*, J. of Pharmaceutical Sciences 2005 94: In Press, Appendix 4). The data was obtained from normal male Sprague-Dawley rats, and it demonstrates the delivery and retention of an active agent to the mediastinal nodes using an avidin/biotin-liposome delivery system.

11. The normal male Sprague-Dawley rat model is a generally accepted model for research concerning avidin/biotin-liposome delivery systems. In this study, avidin and ^{99m}Tc-Blue-Biotin-Liposomes were used. Eight groups of rats, four experimental and four control, were used in two methods of injection to evaluate mediastinal node targeting. Figure 1 in Appendix 4 describes the experimental design and study groups. In each method two different doses (0.5 and 5.0 mg) were studied:

(i) IP/Pleu Method: ^{99m}Tc-blue-biotin-liposomes were injected intraperitoneally and avidin was injected intrapleurally. Experimental groups are denoted IP/Pleu (0.5) and IP/Pleu (5.0), and corresponding control groups are denoted IP/Pleu (0.5)c and IP/Pleu (5.0)c.

(ii) Pleu/IP Method: ^{99m}Tc-blue-biotin-liposomes were injected intrapleurally and avidin was injected intraperitoneally. Experimental groups are denoted Pleu/IP (0.5) and Pleu/IP (5.0), and corresponding control groups are denoted Pleu/IP (0.5)c and Pleu/IP (5.0)c.

12. For the IP/Pleu Method, two experimental groups (IP/Pleu (0.5), IP/Pleu (5.0) were used. For the first group a 0.5 mg dose of ^{99m}Tc-blue-biotin-liposomes (1.67 mg of phospholipid/kg-body wt, 13 MBq) diluted in 1.0 ml saline was injected in the peritoneum. Five minutes later, the same dose of avidin (0.5 mg) diluted in 0.03 ml saline was injected in the pleural space. For the second group, a 5.0 mg dose of the concentrated ^{99m}Tc-blue-biotin-liposomes (16.7 mg of phospholipid/kg-body wt, 12 MBq) diluted in 1.0 ml saline was injected IP. Five minutes later, the same dose of avidin diluted in 0.3 ml was injected in pleural space. Control groups IP/Pleu (0.5)c and IP/Pleu (5.0)c were used using the same doses of ^{99m}Tc-blue-biotin-liposomes, but saline (0.03 and 0.3 ml, respectively) was injected instead of avidin.

13. For the Pleu-IP Method, the first group Pleu/IP (0.5), a 0.5 mg dose of ^{99m}Tc-blue-biotin-liposomes (1.67 mg of phospholipid/kg-body wt, 7 MBq) diluted in 0.03 ml saline was injected in pleural space. Five minutes later, the same dose (0.5 mg) of avidin diluted in 1.0 ml saline was injected IP. For the second dose, group Pleu/IP (5.0), a 5.0 mg dose of ^{99m}Tc-blue-biotin-liposomes (16.7 mg phospholipid/kg-body wt, 29 MBq) dilute in 0.3 ml saline was injected in pleural space. Five minutes later, 5.0 mg avidin diluted in 1.0 ml saline was injected in peritoneum. For control groups Pleu/IP (0.5)c and Pleu/IP (5.0)c, experiments were performed in a similar manner except saline was injected instead of avidin.

14. The results of the four experimental and four control groups show that when no avidin is injected, liposomes leave the injection site (either pleural space or abdominal cavity), move into blood circulation and eventually collect in spleen and liver with minimal accumulation in mediastinal nodes or collecting lymphatics. However, when avidin is injected, accumulation in mediastinal nodes, diaphragm, and collecting lymphatics in the pleura that cover the diaphragm is observed. Table 1 at Appendix 4 provides a summary of these data. Further, these data confirm that the avidin and biotin-liposome containing compositions can be administered at different and non-adjacent sites.

15. I declare that all statements made of my knowledge are true and all statements made on the information are believed to be true; and, further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereupon.

Date: 10-28-05

Beth A. Goins

Beth A. Goins, Ph.D.

CURRICULUM VITAE
Beth A Goins

GENERAL INFORMATION

PERSONAL DATA:

Date of Preparation: 10/06/2005

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San Antonio, TX 78229-3900

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EDUCATION:

Year	Degree	Discipline	Institution/Location
1988	PhD	Biochemistry	University of Tennessee, Knoxville, TN
1985	MS	Biochemistry	University of Tennessee, Knoxville, TN
1980	BS	Chemistry (*Cum Laude*)	King College, Bristol, TN

TRAINING:

Post Doctoral Fellowship

Year	Discipline	Institution/Location
1988-1990	Biochemistry/Biophysics	Naval Research Laboratory Center for Bio/Molecular Science and Engineering, Washington, DC

ACADEMIC APPOINTMENTS:

09/2001 - Present	Associate Professor	The University of Texas Health Science Center at San Antonio, Department of Radiology, San Antonio, TX
09/1995 - 08/2001	Assistant Professor	The University of Texas Health Science Center at San Antonio, Department of Radiology, San Antonio, TX
07/1991 - 08/1995	Clinical Assistant Professor	The University of Texas Health Science Center at San Antonio, Department of Radiology, San Antonio, TX

NON-ACADEMIC APPOINTMENTS:

11/1990 - 06/1991	Scientist II	Geo Center, Inc., Fort Washington, MD
06/1986 - 04/1988	Lab Tech II	Johns Hopkins University, Baltimore, MD

HONORS AND AWARDS:

01/1999	Teacher of the Year: Radiological Sciences Graduate Program
01/1998	Fuji Medical Systems/RSNA: Seed Grant for Radiological Research
01/1993	International Symposium on Blood Substitutes: Young Investigators Award
01/1990	National Research Council Associate Award
01/1986	University of Tennessee Center of Excellence in Science Award
01/1985	University of Tennessee Center of Excellence in Science Award

TEACHING

COURSE BASED TEACHING:

Date	Course Name	Level	Role
01/2005 - 05/2005	RADI5010 Medical Biophysics	Graduate	Course Director
	Preparation Hrs: 40, Student Contact Hrs: 48		
01/2005 - 05/2005	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director
	Preparation Hrs: 16, Student Contact Hrs: 16		
01/2004 - 05/2004	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director
	Preparation Hrs: 16, Student Contact Hrs: 16		

08/2003 - 12/2003	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

01/2003 - 05/2003	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

08/2002 - 12/2002	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

01/2002 - 05/2002	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

08/2001 - 12/2001	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

01/2001 - 05/2001	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

08/2000 - 12/2000	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

01/2000 - 05/2000	RADI5010 Medical Biophysics	Graduate	Course Director

Preparation Hrs: 22, Student Contact Hrs: 33

01/2000 - 05/2000	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

08/1999 - 12/1999	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

01/1999 - 05/1999	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

08/1998 - 12/1998	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

01/1998 - 05/1998	RADI5010 Medical Biophysics	Graduate	Course Director

Preparation Hrs: 20, Student Contact Hrs: 30

01/1998 - 05/1998	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

08/1997 - 12/1997	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

01/1997 - 05/1997	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

08/1996 - 12/1996	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

01/1996 - 05/1996	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

08/1995 - 12/1995	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

01/1995 - 05/1995	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

08/1994 - 12/1994	RADI5090 Sem Radiological Sci	Graduate	Co-Course Director

Preparation Hrs: 16, Student Contact Hrs: 16

OTHER TEACHING:

High School/Junior High School Student Supervision

Date	Description	Institution	# Students
09/2003 - 05/2004	Betty Alajajian	University of Texas Health Science Center at San Antonio	1

Health Careers Scientific Mentoring Program

05/2003 - 08/2003	Jacob Stetler	University of Texas Health Science Center at San Antonio	1

Reagan High School Student

01/2002 - 05/2003	Yang Li	University of Texas Health Science Center at San Antonio	1

Health Careers Scientific Mentoring Program

Membership on Supervising Committee

Date	Description	Institution	# Students
01/2005 - Present	Hua-Hsuan (Irene) Chen, Dissertation	University of Texas Health Science Center at San Antonio	1
08/2004 - 08/2005	Hossein Afrashteh, Dissertation	University of Texas Health Science Center at San Antonio	1

A Back Projection Dosimetry Method For Diagnostic and Orthovoltage X-ray From 40 to 140 KVP for Patients and Phantoms, Radiological Sciences Graduate Program/Radiology Department, Dissertation Awarded August 2005

09/2003 - Present	Max Amurao, Dissertation	University of Texas Health Science Center at San Antonio	1
01/2002 - 08/2003	Ande Bao, Dissertation	The University of Texas Health Science Center at San Antonio	1

Development and Use of 99mTc, 186Re and 188Re-Labeled Liposomes for Nuclear Imaging and Radionuclide Therapy, Radiological Sciences Graduate Program/Radiology Department. Dissertation Awarded August 2003.

01/2000 - 05/2003	William Pramenko, Thesis	The University of Texas Health Science Center at San Antonio	1

Characterization of Radiation Exposure to Hospital Personnel and Members of the General Public from Iodine Abalation Patients, Radiological Sciences Graduate Program/Radiology Department. Thesis Awarded May 2003.

01/1994 - 08/1996	Carl Keener, Dissertation	The University of Texas Health Science Center at San Antonio	1

Algorithm for Predicting Peptide Nonideality in Water, Radiological Science Graduate Program/Radiology Department. Dissertation Awarded August 1996.

01/1994 - 05/1996	Kalpana Kanal Dissertation	The University of Texas Health Science Center at San Antonio	1

Influence of Globular Protein Conformation on Cellular Hydration and MRI Contrast, Radiological Sciences Graduate Program/Radiology Department. Dissertation Awarded May 1996.

Ph.D. Dissertations Directed

Date	Description	Institution	# Students
06/2003 - Present	Cristina Zavaleta	University of Texas Health Science Center at San Antonio	1
06/1999 - 12/2003	Luis A. Medina, Dissertation	University of Texas Health Science Center at San Antonio	1

Evaluation of the Avidin/Biotin-Liposome System Injected in Pleural Space and Peritoneum for Drug Delivery to Mediastinal Lymph Nodes, Radiological Sciences Graduate Program/Radiology Department, Dissertation Awarded December 2003

Post-Doctoral Student Supervision

Date	Description	Institution	# Students
07/2004 - 07/2005	Diagnostic Radiology Resident Physics Lectures	University of Texas Health Science Center at San Antonio	8

Anger Camera-Basic Principles Lecture, SPECT and PET Imaging Lecture

02/2004 - 09/2005	Physics of Nuclear Medicine Lecture Series for Fellows	University of Texas Health Science Center at San Antonio	2

In-vivo Counting Lecture, Anger Camera -Basic Principles Lecture, Anger Camera - Performance Characteristics Lecture, SPECT systems Lecture, Basic Camera QC Lab

Pre-Doctoral Student Supervision

Date	Description	Institution	# Students
06/2004 - Present	Anuradha Soundararajan	University of Texas Health Science Center at San Antonio	1
01/1997 - 08/1997	Trevor Andrews *Radiological Sciences Graduate Program*	University of Texas Health Science Center at San Antonio	1
01/1997 - 06/1997	Ho-Ling Liu *Radiological Sciences Graduate Program.*	University of Texas Health Science Center at San Antonio	1

Undergraduate Student Supervision

Date	Description	Institution	# Students
05/2005 - 08/2005	Jacob Stetler *Baylor University Student*	University of Texas Health Science Center at San Antonio	1
05/2002 - 08/2003	Sam Prater *UTSA Student*	The University of Texas Health Science Center at San Antonio	1
02/2002 - 05/2002	Sergio Calixto *UTSA Student*	The University of Texas Health Science Center at San Antonio	1
06/2000 - 08/2000	Denise Rivera *South Texas Summer Undergaduate Research Fellowship Program.*	University of Texas Health Science Center at San Antonio	1

06/1999 - 08/1999 Allison Thies University of Texas Health Science Center at San Antonio 1

Summer Undergraduate Research Program.

RESEARCH

EXPERTISE:

Aptamers - Use as Targeting Agents

Blood Substitutes - Development and testing of hemoglobin-based liposome oxygen carriers

Imaging - Use non-invasive imaging methods to track agents after injection in the body

Liposome Drug Delivery - Preparation and Testing of Drugs Delivered Using Liposomes

Nanotechnology - Study nanoparticles especially liposomes

Radiolabeling Methods - Develop methods for radiolabeling agents for tracking after injection in the body

PUBLICATIONS: ***('*' indicates Peer Reviewed)***

Abstract

* 1. Amurao, M, Chang, T, Soundararajan, A, Fullerton GD, Goins BA. Spatial Resolution and Geometric Accuracy of a Combined Modality Small Animal Dual-Head Single Photon Emission Computed Tomography/MicroCT 2005. p. 121. (Molecular Imaging and Biology; vol. 7, no. 2).

2. Bao A, Phillips WT, Goins BA, Zheng, X, McGuff HS, Natarajan M, Santoyo, C, Otto RA. Small Animal Imaging and Histopathological Studies on Head and Neck Squamous Cell Carcinoma Xenograft in Nude Rats 2005. p. 175. (Molecular Imaging and Biology; vol. 7, no. 2).

* 3. Sterling JA, Padalecki SS, Grubbs B, Zhao M, Oyajobi B, Goins BA, Mundy GR. Gli2 regulates PTHrP expression and tumor-induced osteolysis by metastatic breast cancer cells 2005. p. S23. (Cancer Treatment Rev; vol. 31).

4. Oyajobi B, Goins BA, Gupta A, Zavaleta CL, Munoz S, Grubbs B, Story B, Wideman C, Garrett IR, Phillips WT, Mundy GR. Imaging tumor burden by [18F]FDG-PET and osteoblast activity by [99mTc]-MDP-SPECT/CT in the 5TGM1 model of myeloma bone disease 2005. p. 901. (Proceedings of the AACR Annual Meeting; vol. 46).

* 5. Zavaleta CL, Phillips WT, Bradley YC, Jerabek PA, Goins BA. Use of microPET to non-invasively monitor the development of an intraperitoneal ovarian xenograft model in nude rats 2005. p. 235P. (J Nucl Med; vol. 46).

* 6. Bao A, Zhao X, Goins B, Otto RA, Phillips WT. The influence of heterogeneous radioactivity distribution on intratumoral radiation dose distribution 2004. p. 438P. (J Nucl Med; vol. 45).

7. Bao A, Goins B, Klipper R, Negrete G, Phillips WT. Novel 186Re-Liposome Labeling Using 186Re-"SNS/S" Complexes for Radionuclide Therapy 2003. p. 306P. (J Nucl Med; vol. 44).

8. Medina LA, Klipper R, Phillips WT, Goins B. Avidin/Biotin-Liposome System for the Delivery of Pharmaceutical Agents to the Mediastinal Nodes and the Diaphragm. 2002. p. S54. (Molecular Imaging and Biology; vol. 4).

9. Medina LA, Klipper R, Phillips WT, Goins BA,. Sentinode Targeting in a Rat Model of Breast Cancer Using 99mTc-Labeled Liposomes Encapsulating Blue Dye. 2002. p. 281P. (J Nucl Med; vol. 43).

10. Phillips WT, Klipper R, Goins B. A Novel Approach for Localization of Sentinel Lymph Nodes Draining the Peritoneum 2001. p. 278P. (J Nucl Med; vol. 42).

11. McManus LM, Phillips WT, Goins BA,Keckler MS, Klipper R, Pinckard RN. Inflammatory Mediators Promote Tissue Sequestration of Radiolabeled Liposomes 2001. p. 332P. (J Nucl Med; vol. 42).

12. Goins BA, Hanes, MA, Klipper R, Phillips WT. Detecting and Monitoring Viral Infections Using 99mTc-Liposomes 2001. p. 333P. (J Nucl Med; vol. 42).

13. Phillips WT, Hanes, MA, Klipper, R, Goins B,. Evaluation of Tc-99m-Liposomes for Detecting and Monitoring Viral Infections 2001. p. 279. (Clin Nucl Med; vol. 25).

14. Medina, LA, Klipper, R, Phillips WT, Goins BA,. The Kinetics and Organ Biodistribution of In-111-Avidin and Tc-99m-Biotin-Liposomes after Injection in the Pleural Space for Mediastinal Lymph Node Targeting 2001. p. 279. (Clin Nucl Med; vol. 25).

15. Goins B, Klipper R, Thies A, Phillips WT. Uptake of Tc-99m-Liposomes in Inflamed Lung of Control and Neutropenic Rats 2000. p. 935. (Eur J Nucl Med; vol. 27).

16. Phillips WT, Klipper R, Goins BA,. Use of Technetium-99m-Labeled Liposomes Encapsulating Blue Dye for Identification of the Sentinel Lymph Node 2000. p. 973. (Eur J Nucl Med; vol. 27).

17. Phillips WT, Klipper R, Blumhardt R, Landry AJ, Andrews T, Liu HL, Goins BA,. Noninvasive Estimation of Lymph Node Retention Efficiency and Percentage Lymph Node Pass Through for Evaluation of Sentinel Node Localization Agents 2000. p. 189P. (J Nucl Med; vol. 41).

* 18. Phillips WT, Klipper R, Goins BA,. Use of 99mTc-Labeled Liposomes Encapsulating Blue Dye for Identification of the Sentinel Lymph Node 2000. p. 229P. (J Nucl Med; vol. 41).

19. Phillips WT, Shriki M, Blumhardt R, Klipper R, Goins BA. Detection of Gastro-intestinal Bleeding with 99m Tc-Radiolabeled Biotin Liposomes and Avidin-Mediated Active Background Reduction. 2000. p. 319P. (J Nucl Med; vol. 41).

20. Medina LA, Klipper R, Phillips WT, Goins BA,. Biotin Liposomes and Avidin: A Novel Method for Targeting the Mediastinal Lymph Nodes 2000. p. 232. (Clin Nucl Med; vol. 24).

* 21. Phillips WT, Shriki M, Blumhardt R, Klipper R, Goins BA,. Detection of Gastro-intestinal Bleeding with Radiolabeled Biotin Liposomes and Avidin-Mediated Active Background Reduction 2000. p. 232. (Clin Nucl Med; vol. 24).

22. Phillips WT, Hanes, MA, Klipper, R, Goins BA. Case Report Demonstrating the Potential Use of 99mTc-Liposomes for Detecting and Monitoring Viral Infections 2000. p. 1086. (Eur J Nucl Med; vol. 27).

23. Goins BA, Andrews, T, Sadeghi, KA, Klipper, R, Lui, HL, Woynarowska BA, Herman TS, Phillips WT. Biodistribution of Technetium-99 (Tc-99m)-Labeled PEG-Liposomes in Tumor-Bearing Rats Following Treatment with Ionizing Radiation Directed to Tumor Site 2000. p. 221-222. (J Liposome Res; vol. 10, no. 2-3).

24. Medina, LA, Klipper, R, Phillips WT, Goins BA. Biotin-Liposomes and Avidin Into the Intrapleural Space: A Novel Method for Targeting the Mediastinal Lymph Nodes 2000. p. 255-256. (J Liposome Res; vol. 10, no. 2-3).

25. Phillips WT, Klipper, R, Goins BA. Novel Method of Greatly Enhanced Delivery of Liposomes to Lymph Nodes 2000. p. 268-269. (J Liposome Res; vol. 10, no. 2-3).

26. Phillips WT, Andrews T, Liu HL, Klipper R, Landry A, Blumhardt R,Goins BA,. Evaluation of Technetium-99m-(Tc-99m)-Labeled Liposomes Versus Tc-99m-Sulfur colloid (SC) and Tc-99m Human Serum Albumin (HSA) for Lymphoscintigraphy in a Rabbit Model 1999. p. 212. (Clin Nucl Med; vol. 24).

27. Phillips WT, Andrews T, Liu HL, Klipper R, Laundry A, Goins BA,. Evaluation of Technetium-99m-(Tc-99m)-Labeled Liposomes Versus Tc-99m-Sulfur Colloid (SC) and Tc-99m-Human Serum Albumin (HSA) for Lymphoscintigraphy in a Rabbit Model 1998. p. 314P. (J Nucl Med; vol. 39).

28. Awasthi V, Goins BA, Klipper R, Phillips WT. Comparison of Technetium-99m Liposomes with Indium-111-White Blood Cells for Localizing Experimental Colitis in a Rabbit Model 1998. p. 59. (J Nucl Med; vol. 39).

29. Goins BA, Awasthi V, Klipper R, Phillips WT. Use of a Technetium-99m-(Tc-99m)-Labeled Biotin-Liposome/Avidin System in a Rabbit Colitis Model to Improve Early Image Detection 1998. p. 125. (J Nucl Med; vol. 39).

30. Awasthi V, Goins BA, Klipper R, Phillips WT. Technetium-99m-Liposomes Versus Idium-111-White Blood Cells for Localizing Experimental Colitis in a Rabbit Model 1998. p. 129. (Clin Nucl Med; vol. 23).

31. Goins BA, Awasthi V, Klipper R, Phillips WT. Evaluation of a Technetium-99m-Labeled

Biotin-Liposome/Avidin System to Improve Early Image Detection in a Rabbit Colitis Model 1998. p. 129. (Clin Nucl Med; vol. 23).

32. Phillips WT, Awasthi V, Goins BA, Klipper R, Blumhardt R. Infection Imaging in Rats by Using Liposomes Labeled with Two Radionuclides-Tc-99m and In-111 (Dual Radiolabeled Liposomes, DRL) 1997. p. 12. (J Nucl Med; vol. 38).

33. Goins BA, Klipper R, Phillips WT. Improved Shelf Stability of Liposomes Labeled with Technetium-99m Using the Glutathione-HMPAO Method 1997. p. 179. (J Nucl Med; vol. 38).

34. Goins BA, Klipper R, Phillips WT. Improved Shelf Stability of Liposomes Labeled with Technetium-99m Using the Glutathione-HMPAO Method 1997. p. 179. (J Nucl Med; vol. 38).

35. Goins BA, Klipper R, Phillips WT. Improved Shelf Stability of Liposomes Labeled with Technetium-99m Using the Glutathione-HMPAO Method 1997. p. 179. (J Nucl Med; vol. 38).

36. Awasthi V, Goins BA, Klipper R, Loredo RA, Korvick, Phillips WT. Comparison of Dual Radiolabeled Liposomes (DRL), Tc-99m-MDP and GA-67 Citrate for Imaging Osteomyelitis in Rabbit Model 1997. p. 182. (J Nucl Med; vol. 38).

37. Goins BA, Aggarwal SK, Klipper R, Awasthi V, Lar\wrence RA, Jenkinson SG, Phillips WT. Technetium-99m-Labeled liposomes Versus Gallium-67 citrate for Detection of Hyperoxia-Induced Lung Inflammation in Rats 1997. p. 185. (J Nucl Med; vol. 38).

38. Phillips WT, Awasthi V, Goins BA, Klipper, Blumhardt R. Infection Imaging by Using Liposomes Labeled with Two Radionuclides - Tc-99m and In-111 (Dual Radiolabeled Liposomes) 1997. p. 201. (Clin Nucl Med; vol. 22).

39. Awasthi V, Goins BA, Loredo RA, Klipper R, Phillips WT. Osteomyelitis Detection by Dual Radiolabeled Liposomes 1997. p. 201. (Clin Nucl Med; vol. 22).

40. Goins BA, Aggarwal SK, Klipper R, Awasthi V, Lawrence RA, Jenkinson SG, Phillips WT. Detection of Hyperozia-induced Lung Inflammation in Rats Technetium-99m-Labeled Liposomes Versus Gallium-67 Citrate 1997. p. 201. (Clin Nucl Med; vol. 22).

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42. Phillips WT, Goins BA, Klipper RW, Bloodworth RC, Meadows RL, Rudolph AS, McManus LM. Intravascular and Hemodynamic Effects of Liposome-Encapsulated Hemoglobin (LEH) in the Rabbit 1996. p. 404A. (Art Cells, Blood Substitutes & Immob Biotech; vol. 24).

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47. Phillips WT, Dixon L, Klipper R, Rudolph AS, Cliff RO, Emch ME, Jerabek PA, Martin CC, Fox, PT, Goins BA,. Use of Cyclotron Produced Oxygen-15 to Study Oxygen Carrying Capacity of Free and Liposome Encapsulated Hemoglobin 1994. p. A145. (Art Cells, Blood Substitutes and Immob Biotech; vol. 22).

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51. Goins BA, Phillips WT, Klipper R, Blumhardt R. Labeling Red blood Cells with Copper-67 1993. p. 173. (Clin Nucl Med; vol. 18).

52. Goins BA, Klipper R, Sanders J, Rudolph AS, Phillips WT. Use of a Tc-99m Liposome Labeling Procedure to Monitor the Circulation Properties of Liposome Encapsulated Hemoglobin in a Rat Shock Model 1993. p. 135. (J Nucl Med; vol. 34).

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54. Goins BA, Klipper R, Phillips WT. Successful Infection Detection in Rats Using a Newly Developed Technetium-99m Liposome Labeling Procedure 1992. p. 984. (J Nucl Med; vol. 33).

55. Goins BA, Klipper R, Rudolph AS, Timmons JH, Blumhardt R, Phillips WT. The Importance of Glutathione in the Formation of Stable Technetium--99m-Labeled Liposomes Using the Lipophilic Chelator Hexamethyl-Propylene Amine Oxime 1992. p. 256. (Clin Nucl Med; vol. 17).

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63. Goins BA, Barisas BG, Freire E. Lateral Diffusion of Gangliosides in DMPC Bilayers 1985. p. 116a. (Biophys J; vol. 47).

Book Chapter

1. Laverman, Peter, Phillips WT, Bao A, Storm, Gert, Goins BA. Radiolabeling of liposomes for scintigraphic imaging In: Gregoriadis, G. Liposome Technology 3rd Edition. Boca Raton, FL: CRC Press; 2006.

2. Phillips WT, Bao A, Medina, Luis A, Goins BA. Liposomes for intracavitary and intratumoral drug delivery In: Gregoriadis, G. Liposome Technology, 3rd Edition. Boca Raton, FL: CRC Press; 2006.

3. Phillips WT, Medina, Luis A, Goins BA. Targeting of liposomes to lymph nodes In: Gregoriadis, G. Liposome Technology, 3rd Edition. Boca Raton, FL: CRC Press; 2006.

4. Goins BA, Phillips WT. Methods for tracking radiolabeled liposomes after injection in the body In: Goins BA, Phillips WT. Liposome Technology, 3rd Edition. CRC Press; 2006.

5. Awasthi V, Goins BA, Phillips WT. Liposome-encapsulated hemoglobin as an artifical oxygen carrier In: Gregoriadis, G. Liposome Technology 3rd Edition. Boca Raton, FL:

CRC Press; 2006.

6. Awasthi V, Goins BA, Phillips WT. Liposome-Encapsulated Hemoglobin: History, Preparation and Evaluation In: Winslow, R.. Blood Substitutes. Oxford, UK: Elsevier; 2005. p. 501 - 513.

7. Goins BA, Phillips WT. Radiolabeled liposomes for imaging and biodistribution studies In: Weissig V, Torchilin V. Liposomes: A Practical Approach. Oxford ,UK: Oxford University Press; 2003. p. 319 - 336.

8. Bao A, Phillips WT, Negrete, G, Klipper, R, Goins BA. 99mTc/186Re/188Re-Liposome Radiolabeling for Nuclear Imaging and Targeted Radionuclide Therapy In: Nicolini M, Mazzi U. Technetium, Rhenium and Other Metals in Chemistry and Nuclear Medicine. Padova, Italy: Servizi Grafici Editorial; 2002. p. 281 - 286.

9. Medina LA, Goins BA. A Novel Option in Nuclear Medicine for Diagnostic Imaging and Internal Therapy In: Zetina LMM, Corral GH. Medical Physics Sixth Mexican Symposium. Melville, NY: AIP Conference Proceedings; 2002. p. 11 - 19.

10. Goins BA, Klipper R, Martin C, Jerabek PA, Khalvati S, Fox PT, Cliff RO, Kwasiborski V, Rudolph AS, Phillips WT. Use of Oxygen-15-Labeled Molecular Oxygen for Oxygen Delivery Studies of Blood and Blood Substitutes. In: Hudetz A and Bruley D. Oxygen Transport to Tissue XX. New York, NY: Plenum Press; 1998. p. 643 - 652.

11. Phillips WT, Goins BA, Klipper R, Cook BG, Martin C, Lemen L, Jerabek PA, Khalvati S, Fox PT, Cliff RO, Kwasiborski V, Rudolph AS. Tissue Oxygen Delivery and Tissue Distribution of Liposome Encapsulated Hemoglobin. In: Tsuchida E. Present and Future Perspectives of Blood Substitutes. Amsterdam, Netherlands: Elsevier Science; 1998. p. 147 - 159.

12. Goins BA, Phillips WT, Klipper, R. Blood-pool Imaging Using Technetium-99m-Labeled Liposomes In: Gottschalk A, Blaufox MD, Neumann RD, Strauss HW, Zubal IG. Yearbook of Nuclear Medicine. St Louis, MO: Mosby; 1998. p. 384 - 386.

13. Phillips WT, Goins BA, Rudolph, AS. Liposome Encapsulated Hemoglobin Biodistribution and Interactions with Platelets Studied with Imaging Radionuclides In: Faist E, Baue AE, Schildberg FW. MOF, MODS and SIRS-Concepts, Clinical Coorelates and Therapy. Lengrich: Pabst Science Publisher; 1996. p. 812 - 818.

14. Phillips WT, Goins BA. Targeted Delivery of Imaging Agents by Liposomes In: Torchilin V. Handbook of Targeted Delivery of Imaging Agents. Boca Raton, FL: CRC Press; 1995. p. 149 - 173.

15. Goins BA, Klipper, R, Rudolph, AS, Cliff, RO, Blumhardt R, Phillips WT. Biodistribution and Imaging Studies of Technetium-99m-Labeled Liposomes in Rats with Focal Infection In: Gottschalk A. Yearbook of Nuclear Medicine. St Louis, MO: Mosby; 1993. p. 2160 - 2168.

16. Cliff, RO, Ligler, F, Goins BA, Hoffman, PM, Spielberg, H, Rudolph, AS. Liposome Encapsulated Hemoglobin: Long Term Storage Stability and In Vivo Characterization In: Chang TMS. Blood Substitutes and Oxygen Carriers. New York: Marcel Dekker; 1993. p. 411 - 418.

17. Phillips WT, Rudolph, AS, Goins BA, Klipper R. Biodistribution Studies of Liposome Encapsulated Hemoglobin (LEH) Studied with a Newly Developed 99mTechnetium Liposome Label In: Chang TMS. Blood Substitutes and Oxygen Carriers. New York: Marcel Dekker; 1993. p. 534 - 537.

18. Goins BA, Rudolph, AS, Ligler, FS. Liposome Encapsulated Hemoglobin: Historical Development of a Blood Substitute In: Biotechnology of Blood. Stoneham, MA: Butterworths; 1991. p. 117 - 125.

19. Rudolph, AS, Goins BA, Ligler, FS, Cliff, RO, Spielberg, H, Hoffman, P, Phillips WT. Liposome Encapsulated Hemoglobin- In Vivo Efficacy of a Synthetic Red Cell Substitute In: Gomezfernandez JC, Chapman D, Packer L. Progress in Membrane Biotechnology. Birkhauser Verlag; 1991. p. 214 - 226.

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Journal Article

* 1. Sou K, Klipper R, Goins BA, Tsuchida E, Phillips WT. Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute J Pharmacol Exp Ther 2005 Feb;312(2):702-709.

* 2. Bao A, Zhao, X, Phillips WT, Woolley FR, Otto RA, Goins BA, Hevezi, JM. Theoretical study of the influence of a heterogeneous activity distribution on intratumoral absorbed dose distribution Medical Physics 2005 Jan;32(1):200-208.

* 3. Medina LA, Calixto SM, Klipper R, Phillips WT, Goins BA. Avidin/Biotin-liposome system injected in the pleural space for drug delivery to mediastinal lymph nodes J Pharm Sci 2004 Oct;93(10):2595-608.

* 4. Awasthi V, Garcia D, Klipper R, Phillips WT, Goins BA. Kinetics of liposome-encapsulated hemoglobin after 25% hypovolemic exchange transfusion Int J Pharm 2004 Sep;283(1-2):53-62.

* 5. Awasthi VD, Garcia D, Klipper R, Goins BA, Phillips WT. Neutral and Anionic Liposome-Encapsulated Hemoglobin: Effect of Postinserted Poly(ethylene glycol)-distearoylphosphatidylethanolamine on Distribution and Circulation Kinetics. J Pharmacol Exp Ther 2004 Apr;309(1):241-248.

* 6. Bao A, Goins BA, Klipper, R, Negrete, G, Phillips WT. Direct 99mTc Labeling of Pegylated Liposomal Doxorubicin (Doxil) for Pharmacokinetic and Non-Invasive Imaging Studies J Pharmacol Exp Ther 2004;308:419-425.

* 7. Medina, LA, Klipper, R, Phillips WT, Goins BA. Pharmacokinetics and biodistribution of [111In] Avidin and [99mTc]-Biotin-Liposomes Injected in the Pleural Space for the Targeting of Mediastinal Nodes Nucl Med Biol 2004;31:41-51.

* 8. Awasthi VD, Garcia D, Goins BA, Phillips WT. Circulation and biodistribution profiles of long-circulating PEG-liposomes of various sizes in rabbits. Int J Pharm 2003 Mar;253(1-2):121-132.

* 9. Awasthi V, Goins BA, McManus LM, Klipper R, Phillips WT. [99mTc] liposomes for localizing experimental colitis in a rabbit model. Nucl Med Biol 2003 Feb;30(2):159-168.

* 10. Awasthi VD, Goins B, Klipper R, Phillips WT. Accumulation of PEG-liposomes in the inflamed colon of rats: potential for therapeutic and diagnostic targeting of inflammatory bowel diseases. J Drug Target 2002 Aug;10(5):419-427.

* 11. Phillips WT, Medina, LA, Klipper, R, Goins BA. A novel approach for the increased delivery of pharmaceutical agents to the peritoneum and associated lymph nodes J Pharmacol Exp Ther 2002;303:11-16.

* 12. Phillips WT, Goins BA. Assessment of Liposome Delivery Using Scintigraphic Imaging J Liposome Res 2002;12:11-16.

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* 14. Goins BA, Phillips WT. The Use of Scintigraphic Imaging During Liposome Drug Development J Pharm Practice 2001;14(4):397-406.

* 15. Phillips WT, Andrews, T, Liu H, Klipper, R, Lnadry AJ, Blumhardt R, Goins BA. Evaluation of [99mTc]liposomes as lymphoscintigraphic agents: comparison with [99mTc] sulfur colloid and [99mTc] human serum albumin Nucl Med Biol 2001;28:435-444.

* 16. Phillips WT, Klipper R, Goins BA. Use of 99mTc-Labeled Liposomes Encapsulating Blue Dye for Identification of the Sentinel Lymph Node J Nucl Med 2001;42(3):446-451.

* 17. Phillips WT, Klipper R, Goins BA. Novel Method of Greatly Enhanced Delivery of Liposomes to Lymph Nodes J Pharmacol Exp Ther 2000;295:309-313.

* 18. Phillips WT, Klipper RW, Awasthi VD, Rudolph AS, Cliff R, Kwasiborski V, Goins BA. Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. J Pharmacol Exp Ther 1999 Feb;288(2):665-670.

* 19. Awasthi V, Goins B, Klipper R, Loredo R, Korvick D, Phillips WT. Imaging experimental osteomyelitis using radiolabeled liposomes. J Nucl Med 1998 Jun;39(6):1089-1094.

* 20. Awasthi VD, Goins B, Klipper R, Phillips WT. Dual radiolabeled liposomes: biodistribution studies and localization of focal sites of infection in rats. Nucl Med Biol 1998 Feb;25(2):155-160.

* 21. Goins BA, Klipper R, Martin C, Jerabek PA, Khalvati S, Fox, PT, Cliff RO, Kwasiborski V, Rudolph AS, Phillips WT. Use of oxygen-15-labeled molecular oxygen for oxygen delivery studies of blood and blood substitutes. Adv Exp Med Biol 1998;454:643-652.

* 22. Goins BA, Phillips WT, Klipper, R. Repeat Injection Studies of Technetium-99m-Labeled PEG-Liposomes in the Same Animal. J Liposomes Res 1998;8:265-281.

* 23. Phillips WT, Lemen L, Goins B, Rudolph AS, Klipper R, Fresne D, Jerabek PA, Emch ME, Martin C, Fox PT, McMahan CA. Use of oxygen-15 to measure oxygen-carrying capacity of blood substitutes in vivo. Am J Physiol 1997 May;272(5 Pt):2492-2499.

* 24. Phillips WT, Klipper BS, Fresne D, Rudolph AS, Javors MA, Goins BA. Platelet Reactivity with Liposome Encapsulated Hemoglobin in the Rat. Experimental Hematology 1997;25:1347-1356.

* 25. Goins BA, Phillips WT, Klipper R, Rudolph AS. Role of Complement in Rats Injected with Liposome-Encapsulated Hemoglobin J Surg Res 1997;68:99-105.

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* 27. Goins BA, Phillips WT, Klipper R. Blood Pool Imaging Using Technetium-99m-Labeled Liposomes J Nucl Med 1996;37:1374-1379.

* 28. Goins BA, Klipper R, Sanders J, Cliff RO, Rudolph AS, Phillips WT. Physiological Responses, Organ Distribution and Circulation Kinetics in Anesthetized Rats After Hypovolemic Exchange Transfusion with Technetium-99m-Labeled Liposome-Encapsulated Hemoglobin SHOCK 1995;4:121-130.

* 29. Goins BA, Ligler FS, Rudolph AS. Inclusion of ganglioside GM1 into liposome encapsulated hemoglobin does not extend circulation persistence at clinically relevant doses. Artif Cells Blood Substit Immobil Biotechnol 1994;22(1):9-25.

* 30. Goins BA, Klipper R, Rudolph AS, Phillips WT. Use of Technetium-99m-Labeled Liposomes in Tumor Imaging J Nucl Med 1994;35:1491-1498.

* 31. Rudolph AS, Cliff RO, Klipper R, Goins BA, Phillips WT. Circulation Persistence and Biodistribution of Lyophilized Liposome Encapsulated Hemoglobin: An Oxygen Carrying Resuscitative Fluid. Crit Care Med 1994;22:142-150.

* 32. Goins BA, Klipper R, Sanders J, Rudolph AS, Phillips WT. Circulation Profile of Technetium-99m Labeled Liposome Encapsulated Hemoglobin in a 10% or 50% Rat Hypovolemic Shock Model Biomat Art Cells Immob Biotech 1994;22:909-915.

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Infection. J Nucl Med 1993;34(12):2160-2168.

* 35. Cliff RO, Ligler F, Goins BA, Hoffmann PM, Spielberg H, Rudolph AS. Liposome encapsulated hemoglobin: long-term storage stability and in vivo characterization. Biomater Artif Cells Immobilization Biotechnol 1992;20(2-4):619-626.

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* 39. Goins BA, Freire E. Thermal stability and intersubunit interactions of cholera toxin in solution and in association with its cell-surface receptor ganglioside GM1. Biochemistry 1988 Mar;27(6):2046-2052.

* 40. Goins B, Masserini M, Barisas BG, Freire E, Goins BA. Lateral diffusion of ganglioside GM1 in phospholipid bilayer membranes. Biophys J 1986 Apr;49(4):849-856.

* 41. Goins BA, Freire E. Lipid phase separations induced by the association of cholera toxin to phospholipid membranes containing ganglioside GM1. Biochemistry 1985 Mar;24(7):1791-1797.

PRESENTATIONS:

06/2005 Indium-111 platelet imaging studies for monitoring transient thrombocytopenia after infusion of various liposome encapsulated hemoglobin (LEH) formulations, International Society of Blood Substitutes, Providence, RI (Sole Presenter)

04/2005 Nanotechnology Now & the Future, 50th Annual Meeting, Southwestern Chapter Society of Nuclear Medicine, San Antonio, TX (Invited Speaker)

Details: *Winfield Evans Lecture*

12/2003 Liposomes Labeled with Diagnostic or Therapeutic Radionuclides for Local/Regional Delivery, Sixth International Conference Liposome Advances: Progress in Drug and Vaccine Delivery, London, UK (Sole Presenter)

04/2002 Radiolabeled Liposomes for Medical Imaging and Assessment of Liposome Delivery Systems, Particles 2002, Orlando, FL (Sole Presenter)

02/2002 Overview of Liposome Chemistry for Targeted Drug Delivery and Imaging Applications., Chemistry Department, University of Texas at San Antonio, San Antonio, TX (Invited Speaker)

12/2001 Detecting and Monitoring Viral Infections Using 99mTc-Liposomes, Liposome Advances: Progress in Drug and Vaccine Delivery, London, UK (Co-Presenter)

06/2001 Prolonged Tissue Accumulation of Radiolabeled Liposomes During Persistent Inflammation, ISORBE, Toronto, CA (Co-Presenter)

06/2001 Use of Oxygen-15 as a Tool for the Assessment of Oxygen Delivery and the Effects of Hemodynamic Alterations on Oxygen Extraction, Combat Fluid Resuscitation 2001, Bethesda, MD (Co-Presenter)

10/2000 The Use of Scintigraphic Imaging During Liposome Drug Development, Society of Nuclear Imaging in Drug Development 2000 Symposium, Bethesda, MD (Sole Presenter)

12/1999 Accumulation of Technetium-99m-Labeled Liposomes in Control and Neutropenic Rats with Hyperoxia-Induced Lung Inflammation., Liposome Advances: Progress in Drug and Vaccine Delivery, London, UK (Co-Presenter)

12/1999 Novel Method of Greatly Enchanced Delivery of Liposomes to Lymph Nodes., Liposome Advances: Progress in Drug and Vaccine Delivery, London, UK (Co-Presenter)

12/1999 Tc-99m-Liposomes, Research Staff, Nycomed-Amersham, Amersham, UK (Invited Speaker)

01/1999 Radiolabeled Liposomes, Nuclear Medicine Department, St. Luke's Episcopal Hospital, Houston, TX (Invited Speaker)

11/1998 Mechanism of Liposome Accumulation in Injured Lung: An Example of the Use of 99mTc-Liposomes in Nuclear Medicine Imaging., First Congress Iberolatinoamericano y Del Caribe De Fisica Medica, Mexico City, MX (Co-Presenter)

11/1998 Tc-99m-Liposomes, Research Staff, Nycomed-Amersham, Amersham, UK (Invited Speaker)

03/1998 Use of Liposomes in Cancer Diagnosis and Treatment, Radiation Oncology Research Seminar, University of Texas Health Science Center at San Antonio, San Antonio, TX (Invited Speaker)

10/1997 Imaging Disease Processes Using Technetium-99m-Labeled Liposomes, American Nuclear Society Health Physics Society Meeting, Student Section, College of Engineering, Texas A&M University, College Station, TX (Invited Speaker)

09/1997 Tissue Oxygen Delivery and Tissue Distribution of Liposome Encapsulated Hemoglobin, VII International Symposium on Blood Substitutes, Tokyo, JA (Co-Presenter)

09/1997 Oxygen Delivery Studies of Blood and Blood Substitutes Using Oxygen-15-Labeled Molecular Oxygen., VII International Symposium on Blood Substitutes, Tokyo, JA (Co-Presenter)

08/1997 Use of Oxygen-15-Labeled Molecular Oxygen for Oxygen Delivery Studies of Blood and Blood Substitutes, 25th Annual Meeting of the International Society on OxygenTransport to Tissue, Milwaukee, WI (Sole Presenter)

03/1997 Surface Modification of Liposome-Encapsulated Hemoglobin with Polyethylene Clycol Significantly Increases Circulation Persistence., Current Issues in Blood Substitutes Research and Development-1997, San Diego, CA (Sole Presenter)

08/1996 Oxygen Carrying Capacity and Tissue Oxygen Delivery of Liposome Encapsulated Hemoglobin Using Oxygen-15-Labeled Molecular Oxygen, VI International Symposium on Blood Substitutes, Montreal, CA (Co-Presenter)

08/1996 Targeting of Glutathione Containing Liposomes in Rat Lung Exposed to 100% Oxygen, South Texas Chapter of the Oxygen Society, San Antonio, TX (Invited Speaker)

03/1996 Use of Oxygen-15-Labeled Oxygen to Study Lung Uptake and Tissue Delivery of Liposome Encapsulated Hemoglobin., Current Issues in Blood Substitute Research and Development, San Diego, CA (Co-Presenter)

03/1996 Role of Complement in Transient Thrombocytopenia Elicited by LEH in the Rat: Indium-111-Labeled Platelet Studies., Current Issues in Blood Substitute Research and Development, San Diego, CA (Sole Presenter)

03/1994 Liposome Encapsulated Hemoglobin (LEH) Biodistribution and LEH/Platelet Interactions Studied with Imaging Radionuclides., 3rd International Congress on the Immune Consequences of Trauma, Shock and Sepsis Mechanisms and Therapeutic Approaches, Munich, GM (Co-Presenter)

12/1993 Use of a New 99mTc Liposome Labeling Method for Diagnostic Imaging and Drug Targeting Applications, Liposomes in Drug Delivery: The Nineties and Beyond, London, UK (Co-Presenter)

RESEARCH GRANTS:

Federal

Project #: 121668
Funding Agency: National Cancer Institute
Title: Radiofrequency Ablation and Rhenium Liposomes for Solid Tumor Therapy
Status: Active
Period: 09/2005 - 09/2007
Role: Co-Investigator
Total Costs: $486,754.00

Grant Detail:

Project #: 117991
Funding Agency: Office of Naval Research
Title: Development and Assessment of Long Circulating Oxygen Carriers
Status: Active
Period: 11/2003 - 09/2005
Role: Co-Investigator
Total Costs: $756,083.00
Grant Detail:

Project #:
Funding Agency: Office of Naval Research
Title: Development and Assessment of Long Circulation Oxygen Carriers.
Status: Complete
Period: 05/2000 - 09/2003
Role: Co-Investigator
Total Costs: $1,052,304.00
Grant Detail:

Project #:
Funding Agency: NIH
Title: Platelet Interaction and Efficacy Studies of LEH
Status: Complete
Period: 08/1999 - 07/2000
Role: Co-Investigator
Total Costs: $624,066.00
Grant Detail:

Project #:
Funding Agency: Naval Medical Research and Development Command
Title: In Vivo Red Blood Cell Biodistribution and Circulation Persistence
Status: Complete
Period: 09/1997 - 03/1999
Role: Co-Investigator
Total Costs: $50,000.00
Grant Detail:

Private

Project #:
Funding Agency: Nycomed Amsham

Title: Tc-99m Labeled Liposomes
Status: Active
Period: 04/1999 - 12/2001
Role: Co-Investigator
Total Costs: $270,000.00
Grant Detail:

Project #:
Funding Agency: RSNA Research and Education Fund - Seed Grant
Title: Fluorine-18-Labeled Liposomes for Inflammation Imaging
Status: Complete
Period: 07/1998 - 03/2000
Role: Principal Investigator
Total Costs: $24,691.00
Grant Detail:

Project #:
Funding Agency: Nycomed Amersham
Title: Liposomes for Diagnostic Imaging
Status: Complete
Period: 03/1998 - 12/2000
Role: Principal Investigator
Total Costs: $16,000.00
Grant Detail:

Project #:
Funding Agency: American Heart Association - Texas Affiliate
Title: Mechanism of Intravascular Lung Accumulation of Liposomes in a Rat
Status: Complete
Period: 07/1997 - 06/2001
Role: Principal Investigator
Total Costs: $172,574.00
Grant Detail:

State

Project #:
Funding Agency: Texas Higher Education Coordinating Board (Texas Advanced Research Program)
Title: Rapid Screening Method Using Aptamer-Liposomes for Customized Cancer Treatment
Status: Complete
Period: 01/2002 - 12/2003

Role: Principal Investigator
Total Costs: $132,828.00
Grant Detail:

Project #:
Funding Agency: UTHSCSA Children's Cancer Research Center
Title: Aptamer-Liposomes for Diagnosis of Neuroblastoma
Status: Complete
Period: 10/2001 - 09/2002
Role: Principal Investigator
Total Costs: $52,540.00
Grant Detail:

Project #:
Funding Agency: UTHSCSA Institutional Grant
Title: Targeting of Myocardial Infarct with Tc-99-Liposomes
Status: Complete
Period: 09/1996 - 08/1997
Role: Principal Investigator
Total Costs: $5,513.00
Grant Detail:

SERVICE

SERVICE TO THE INSTITUTION:

Dates	Type	Description	Role
01/2000-Present	Department	Department Animal Research Officier	Representative

SERVICE TO THE PROFESSION:

Dates	Type	Description	Role
03/2005-12/2006	International	Journal of Liposome Research	Editorial Board Member
04/2004-04/2007	Local	Society of Nuclear Medicine Southwestern Chapter	Secretary
01/2004-12/2005		Journal of Pharmacology and Experimental Therapeutics	Reviewer

Manuscript Reviewer

01/2004-01/2004		Journal of Pharmaceutical Sciences	Reviewer
Manuscript Reviewer			
01/2003-01/2003		Journal of Nuclear Medicine	Reviewer
Manuscript Reviewer			
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A Novel Approach for the Increased Delivery of Pharmaceutical Agents to Peritoneum and Associated Lymph Nodes

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ABSTRACT

A novel method for prolonging the retention of liposomes in the peritoneum while increasing liposome deposition in lymph nodes that drain the peritoneum is described. An aliquot (1 ml) of technetium-99m (^{99m}Tc)-biotin-liposomes encapsulating blue dye was injected intraperitoneally in rats. Thirty minutes after administration of the ^{99m}Tc-blue-biotin-liposomes, five rats (experimental) were administered avidin (5 mg) intraperitoneally, whereas the remaining five rats served as controls. Scintigraphic images were acquired at baseline and 1 and 24 h after the liposome injection followed by a tissue biodistribution study. Images at 24 h clearly demonstrated very different distributions between the experimental and control animals. In experimental rats, most of the activity was visualized in the abdominal region, and in abdominal and mediastinal lymph nodes. The percentage of the injected dose (% ID) in the blood was significantly higher in the control group than in the experimental group (14.0 ± 1.7 versus 0.17 ± 0.03%; $P < 0.001$). The % ID in the spleen was also significantly greater for controls (23.3 ± 3.9%) compared with the experimental group (0.78 ± 0.8%; $P = 0.001$). Significant ^{99m}Tc activity was detected in blue-stained abdominal nodes (4.7%) and mediastinal nodes (2.3%) from the experimental animals, whereas no blue-stained nodes were detectable in the control animals. The intraperitoneal biotin-liposome/avidin delivery system described in this study could potentially be used for delivery of liposome-encapsulated drugs to disease processes that become disseminated in the peritoneum such as metastatic ovarian, gastric, and colorectal cancer, as well as infectious peritonitis.

There has been a long-standing interest in the local delivery of pharmaceutical and biological agents into the peritoneum for the treatment of peritoneal diseases (Weisberger et al., 1955; Dedrick et al., 1978; Parker et al., 1981a; Markman et al., 1992; Schneider, 1994; Markman, 2001; De Bree et al., 2002). The basic goal of this treatment approach is to increase either the local drug concentration or the duration of drug exposure to a peritoneal disease process while decreasing systemic drug toxicity. Although several recent investigations have examined the efficacy of intraperitoneally delivered antibiotics, therapeutic radionuclides, and genes (Kresta et al., 1993; Meredith et al., 1996; Reimer et al., 1999), the majority of the research in this area has been conducted with intraperitoneally administered chemotherapeutic agents for the treatment of peritoneal carcinomatosis and ovarian cancer (Howell et al., 1991; Markman, 1998). Studies with chemotherapeutic agents administered intraperitoneally have yielded encouraging results for the treatment of ovarian cancer (Alberts et al., 1996; Markman, 2001). A recent consensus statement from specialists in the field of ovarian cancer recommends that intraperitoneal therapy with chemotherapy and/or biological agents be pursued as a legitimate area of research (Alberts et al., 1996; Berek et al., 1999). Reevaluation of the role of intraperitoneal chemotherapy for treatment of ovarian cancer has also recently been suggested (Kaye, 2001).

The normal pathway of drug clearance from the peritoneum is either through direct absorption across the peritoneal membrane or by drainage into the lymphatic system through absorption by the diaphragmatic stomata (Zakaria et al., 1996). Although most intraperitoneally delivered drugs are rapidly cleared from the peritoneal fluid, this method of administration can achieve much higher peak concentrations in the peritoneal fluid compared with the same drug administered intravenously (20-fold higher for cisplatin and carboplatin to as high as 1000-fold for Taxol) (Howell et al., 1991; Markman et al., 1992). Although these drug levels quickly equilibrate with plasma after termination of the peritoneal infusion, transiently elevated peritoneal drug levels provide a significant therapeutic advantage. Unfortunately, this rapid clearance from the peritoneum counteracts the advantages derived from intraperitoneal infusion. One approach to

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ABBREVIATIONS: % ID, percentage of injected dose; GSH, glutathione; PBS, phosphate-buffered saline; HMPAO, hexamethylpropyleneamine oxime; ^{99m}Tc, technetium-99m.

improve peritoneal drug delivery is to encapsulate the drug in a liposome (Parker et al., 1981b; Rosa and Clementi, 1983).

Liposomes are spontaneously forming lipid spheres that have been designed to encapsulate a variety of pharmacological agents (Lasic, 1996). By encapsulating a drug in a liposome, pharmacokinetics and volume of distribution of the drug can be greatly altered without diminishing its therapeutic efficacy. Intraperitoneal administration of a drug encapsulated within a liposome effectively shifts the drug clearance pathway away from direct absorption through the peritoneal membrane into clearance by lymphatic drainage. This slows the clearance of liposome encapsulated drugs from the peritoneum compared with the free drug and has been shown to prolong the contact of tumor cells with drugs, increasing antitumor activity (Sadzuka et al., 2002).

Although intraperitoneally administered conventional liposomes are more slowly cleared from the peritoneal fluid than free drug, the clearance of liposomes from the peritoneum is still fairly rapid (peritoneal clearance half-life of 1–6 h) (Parker et al., 1981b; Sadzuka et al., 2000), and the retention of liposomes in lymph nodes receiving lymph draining from the peritoneum is relatively low [percentage of the injected dose <1% (% ID) per lymph node] (Parker et al., 1981b). This low lymph node retention occurs because the majority of intraperitoneally administered liposomes returns to the systemic circulation by passing through abdominal and mediastinal lymph nodes. This minimal retention of conventional liposomes in lymph nodes has been described previously for liposomes administered subcutaneously (Oussoren et al., 1997; Phillips et al., 2001a).

We have recently described a novel method of greatly increasing the retention of subcutaneously injected liposomes in lymph nodes (Phillips et al., 2000, 2001b). This method increases the retention of liposomes in the primary lymph nodes from 1 to 2 to 12%. This method consists of liposomes coated with biotin that are subcutaneously injected, followed by an adjacent injection of avidin. The avidin causes aggregation of the liposomes, which become entrapped in the next encountered lymph node. In this article, we extend this basic methodology to peritoneal drug delivery as a means of prolonging the retention of liposome-encapsulated drugs in the peritoneum while greatly increasing their deposition in lymph nodes that receive lymphatic drainage from the peritoneum. This study was conducted with biotin-coated liposomes that encapsulated blue dye and were labeled with technetium-99m (^{99m}Tc) to enable both visual identification of lymph nodes as well as scintigraphic imaging and quantitation of liposome distribution.

Materials and Methods

Liposome Preparation and Characterization. Liposomes were comprised of a 50.5:45:2.5:2 M ratio (total lipid) of distearoyl phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL)/cholesterol (Calbiochem, San Diego, CA)/*N*-biotinoyl distearoyl phosphoethanolamine (Northern Lipids; Vancouver, BC, Canada)/α-tocopherol (Aldrich Chemical Co., Milwaukee, WI). Liposomes were prepared as described previously (Phillips et al., 2001b) in a laminar flow hood using aseptic conditions. A dried film was formed by mixing the lipid ingredients in chloroform and then removing the chloroform by rotary evaporation and vacuum desiccation for at least 4 h. The dried lipid film was rehydrated in 300 mM sucrose (Sigma-Aldrich, St. Louis, MO) in sterile water at a total lipid concentration of 120 μmol/ml and lyophilized overnight. The resultant lyophilized powder was then rehydrated with 200 mM reduced glutathione (GSH) (Sigma-Aldrich) and 10 mg/ml patent blue violet dye (CI 42045; Sigma-Aldrich) in Dulbecco's phosphate-buffered saline (PBS), pH 6.3, at a final total lipid concentration of 120 μmol/ml. Immediately before extrusion, the lipid suspension was diluted to 40 μmol/ml with 100 mM GSH and 10 mg/ml blue dye in PBS, pH 6.3, containing 150 mM sucrose, and extruded through a series (2 μm, two passes; 400 nm, two passes; and 100 nm, five passes) of polycarbonate filters (Lipex, Vancouver, BC, Canada) at 55°C. Extruded liposomes were washed three times in PBS, pH 6.3, containing 75 mM sucrose and centrifuged at 45,000 rpm for 45 min in an ultracentrifuge (Ti60 rotor; Beckman Coulter, Inc., Fullerton, CA) to remove any unencapsulated sucrose, GSH, and blue dye. The final liposome pellet was reconstituted in 300 mM sucrose/PBS to a total lipid concentration of approximately 60 μmol/ml and stored at 4°C until needed.

The size distribution of two separate samples of liposomes was determined using an argon laser (λ of 488 nm), and a BI-8000AT digital Autocorrelator and software (Brookhaven Instruments, Holtsville, NY). All measurements were collected at 20°C and a 90° scattering angle. The time correlation function was evaluated with the CONTIN Laplace inversion method. The size distribution histogram was monomodal with a mean diameter of 129.8 nm (range of 115.3 to 148.9 nm; relative variance, 0.004; skew, 0.241; and root mean square error, 1.12×10^{-3}). Phospholipid concentration was determined to be 29 mM using the Stewart assay (Stewart, 1980). The intraliposomal concentration of blue dye was determined spectrophotometrically to be 0.15 mg/ml (Hirnle et al., 1988; Phillips et al., 2001b).

Liposome Labeling. Liposomes were labeled with ^{99m}Tc as described previously (Phillips et al., 1992). A commercial kit of the lipophilic chelator hexamethylpropyleneamine oxime (HMPAO, Ceretec; Amersham Health, Princeton, NJ) was reconstituted with 5 ml of saline containing 370 MBq of ^{99m}Tc-pertechnetate. The kits were checked for the percentage of lipophilic HMPAO using the three-step paper chromatography system as outlined in package insert. An aliquot (1 ml) of ^{99m}Tc-HMPAO was added to a concentrated suspension of liposomes encapsulating GSH and blue dye (1 ml; phospholipid concentration 29 mM), and incubated at room temperature for 30 min. Labeling efficiencies were determined from the ^{99m}Tc activity associated with the ^{99m}Tc-liposomes before and after Sephadex G-25 column separation with a dose calibrator (model Mark 5; Radix, Houston, TX). For three separate labeling experiments, the labeling efficiency was 92.8 ± 2.4%.

Imaging Studies. All animal studies were conducted under the National Institutes of Health Animal Use and Care guidelines and approved by our Institutional Animal Care Committee. Imaging studies were performed on two groups of male Sprague-Dawley rats (200–300 g), experimental (n = 5) and control (n = 5). Rats were anesthetized with ketamine/xylazine (both from Vedco, St. Joseph, MO) (50:10 mg/kg, v/v) in the thigh muscle. An aliquot (1 ml) of ^{99m}Tc-blue-biotin-liposomes was diluted with 1 ml of saline, and this volume (2 ml; 32.7 mg of phospholipid/kg; 43 MBq) was injected into the peritoneum in both experimental and control rats. The volume of liposomes chosen for this study was determined from pilot experiments. Without dilution of the liposomes, we observed less movement into the lymphatics and more liposome aggregates in the abdomen. Anterior whole body dynamic (1 min) images were acquired with rats in a prone position lying on top of the camera face (64 × 64 word image matrix with a zoom of 1.66) using a Dyna 4 gamma camera (Picker, Cleveland, OH) interfaced to a Pinnacle computer (Medasys, Miami, FL) for 60 min after administration of the ^{99m}Tc-liposomes. Thirty minutes after administration of the ^{99m}Tc-blue-biotin-liposomes, the experimental rats were administered avidin (5 mg in 1 ml of saline) (Sigma-Aldrich) intraperitoneally. The timing of the avidin injection was determined from previous pilot studies. At the end of 1 h, the rats were allowed to recover from anesthesia and

housed for the night. At 24 h, animals were again anesthetized as described previously, and anterior static images were acquired for 1 min in a 64 × 64 image matrix.

Image Analysis. A region of interest was placed over the whole animal on the 1-min baseline image and on the 24-h static image to determine the percentage of activity that had cleared from the body of each rat through urine or feces. The 24-h counts were decay corrected and the percentage of baseline counts remaining in the animal was calculated.

Biodistribution Studies. After the imaging study, rats were euthanized by cervical dislocation. Tissues were harvested, weighed, and counted for radioactivity (multichannel analyzer; Packard Bioscience, Meridan, CT). The % ID per organ was calculated by comparison with a standard aliquot of ^{99m}Tc-blue-biotin-liposomes.

Statistical Analysis. Values are reported as mean ± S.E. Statistical analysis was performed using Excel (Microsoft, Redmond, WA) software for a MacIntosh computer (Apple, Cupertino, CA). Statistical differences in the % ID per organ between the experimental and control groups were determined using an unpaired Student's t test. The acceptable probability for a significant difference between means was $P < 0.05$.

Results

Figure 1A shows the 24-h whole body scintigraphic images of four different experimental rats (top images) compared with four different control rats (bottom images). Figure 1B shows an enlarged image of an experimental rat compared with a control rat. These images clearly demonstrate the very different biodistributions between the experimental and the control animals. Also, the increased accumulation of ^{99m}Tc activity in the abdominal nodes and mediastinal nodes of experimental rats is evident. All the control animals have similar organ distributions with a high concentration of activity in the spleen, which is the organ with the greatest liposome uptake in the control animals. Experimental rats given avidin have virtually no uptake in the spleen. In experimental rats, most of the activity was confined to the abdominal region, although the distribution on the images was more variable than in the control animals. From the images, it can be seen that all experimental animals had activity in the mediastinal nodes. Image analysis of the whole animal body revealed that 81.9 ± 1.5% of the dose was still retained in the body of the experimental animals compared with only 72.8 ± 0.20% retained in the body of control animals after 24 h ($P = 0.001$), consistent with a faster clearance from the body of the ^{99m}Tc in the control animals compared with the rats administered avidin.

The tissue biodistribution results are shown in Table 1. Blood activity is significantly higher ($P < 0.001$) in the control group (14.0 ± 1.7%) compared with the experimental group (0.17 ± 0.03%). The % ID measured in the spleen is also significantly higher ($P < 0.01$) for the control group (23.3 ± 3.9%) compared with the experimental group (0.78 ± 0.8%). Significant amounts of activity were also detected in blue-stained abdominal nodes (4.7%) and blue-stained mediastinal nodes (2.3%) in the experimental animals (Table 1). Photographs of the mediastinum and abdomen in an experimental animal at 24-h necropsy after ^{99m}Tc-blue-biotin-liposome administration are shown in Fig. 2, A and B. The blue staining of the mediastinal nodes and the abdominal node is clearly demonstrated in these photos. No blue-stained nodes were detectable in the control animals. The activities in the kidneys and lungs were also significantly higher in the control animals compared with the experimental animals. The only organ with substantial uptake in the experimental animals was the liver, which had 7.7% ID compared with 9.8% ID in the control animals. The total activity in the blood and major organs (liver, spleen, kidneys, lungs, and blood) was much greater in the control animals compared with the experimental animals (51.7 versus 9.6%), indicating that the ^{99m}Tc-blue-biotin-liposomes in the absence of avidin were easily cleared from the peritoneum and associated lymph nodes.

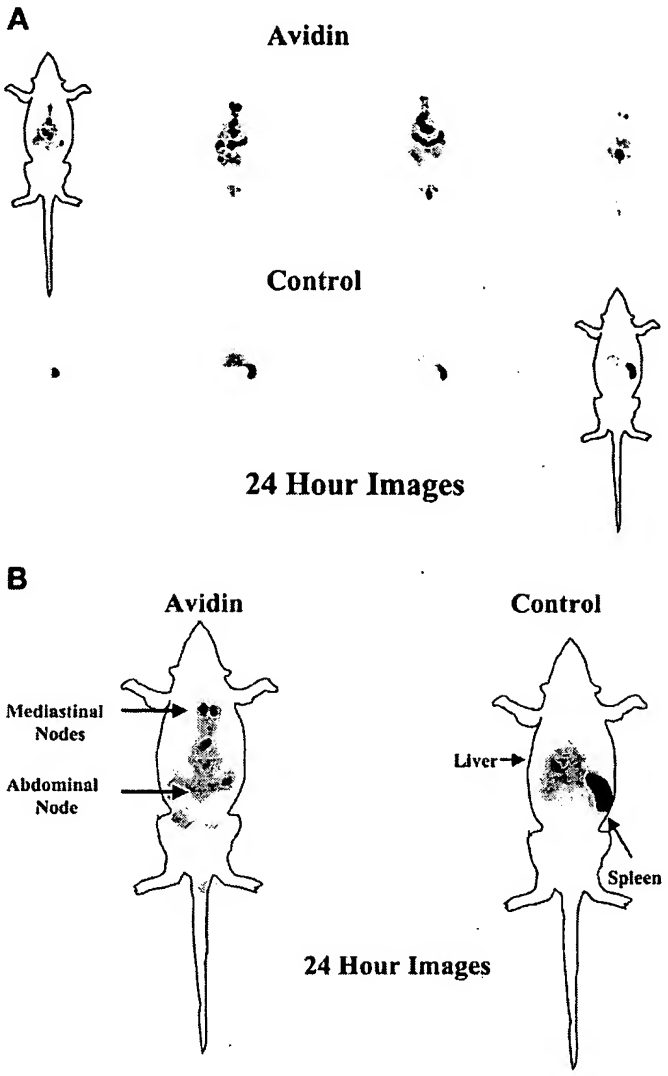

Fig. 1. A, scintigraphic images of four experimental rats (top row) with avidin and four control rats (bottom row) acquired 24 h postinjection. Note the very different distributions in the experimental animals, which have much greater activity in the peritoneal region and in the abdominal and mediastinal lymph nodes, without activity visualized in the spleen. The control animals have consistently high spleen uptake with virtually no activity remaining in the peritoneum. B, single enlarged scintigraphic images of an experimental rat (avidin) compared with a control rat for a more detailed comparison.

Discussion

Previous studies have shown that free drugs administered into the peritoneum are rapidly cleared by absorption through the peritoneal lining. In a clinical study of free

TABLE 1
Biodistribution of ^{99m}Tc-blue-biotin-liposomes at 20 h after i.p. injection in rats
Values are mean ± S.E. $n = 5$.

Organ	Experimental (+Avidin)	Control (−Avidin)
	% ID/organ	
Spleen	0.8 ± 0.2	23.3 ± 3.9**
Blood	0.2 ± 3.0	14.1 ± 1.7***
Abdominal nodes	4.7 ± 3.0	—[a]
Mediastinal nodes	2.3 ± 0.6	—[a]
Liver	7.7 ± 1.9	9.8 ± 0.7
Kidney	0.8 ± 0.2	3.9 ± 0.4***
Lung	0.1 ± 0.1	0.5 ± 0.1*

[a] Could not be identified and removed for counting because blue staining of nodes did not occur. Only minimal accumulation of ^{99m}Tc-blue-biotin-liposomes in these nodes could have occurred.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Depicted P values (two-tailed unpaired t test) refer to differences between experimental and control group.

cisplatin administered by continuous hyperthermic peritoneal perfusion, only 27% of the administered cisplatin remained in the peritoneal fluid at the end of a 90-min infusion (Cho et al., 1999). Most of the cisplatin dose rapidly entered the systemic circulation by direct absorption through the peritoneal membrane.

The encapsulation of drugs in liposomes for intraperitoneal administration has several potential advantages. First, direct local toxicity of the chemotherapeutic agent may be attenuated because of encapsulation of the drug inside the protective lipid bilayer of the liposome. This is important because the dose-limiting toxicity of many intraperitoneally administered drugs is abdominal pain from direct peritoneal irritation (Markman, 2001). Liposome encapsulation has been shown to have reduced local toxicity compared with free drug when extravasated into tissue (Madhavan and Northfelt, 1995).

Second, the encapsulated drug is blocked from rapid direct absorption through the peritoneal lining, resulting in increased time for the liposome-encapsulated drug to reach tumor cells, while the encapsulated drug is slowly cleared through the lymphatics. Many studies have clearly demonstrated that the pharmacokinetics of liposome-encapsulated drugs administered intraperitoneally is very different from the same nonencapsulated drug administered intraperitoneally (Parker et al., 1981; Rosa and Clementi, 1983; Sadzuka et al., 2000). Slow removal of liposomes from the peritoneal cavity seems to provide a sustained release of drug from the liposomes into the peritoneal cavity. In one study in which liposomes encapsulating cefoxitin were administered intraperitoneally, the release of cefoxitin from the liposome complex was estimated to be well in excess of the maximum inhibitory concentration (Kresta et al., 1993). Similar findings were also described in a model of peritoneally disseminated cancer in which doxorubicin encapsulated in liposomes was considered to be slowly released in the abdominal cavity from disrupted liposomes (Sadzuka et al., 2000).

Third, increased lymph node targeting is possible because liposome-encapsulated drugs are cleared through the lymphatic vessels with at least a portion of the administered drug being deposited in the lymph nodes, where it degrades and is slowly released from the liposome in high concentration (Parker et al., 1981b; Hirano and Hunt, 1985). The avidin/biotin-liposome intraperitoneal delivery system should have the same advantages as described above for

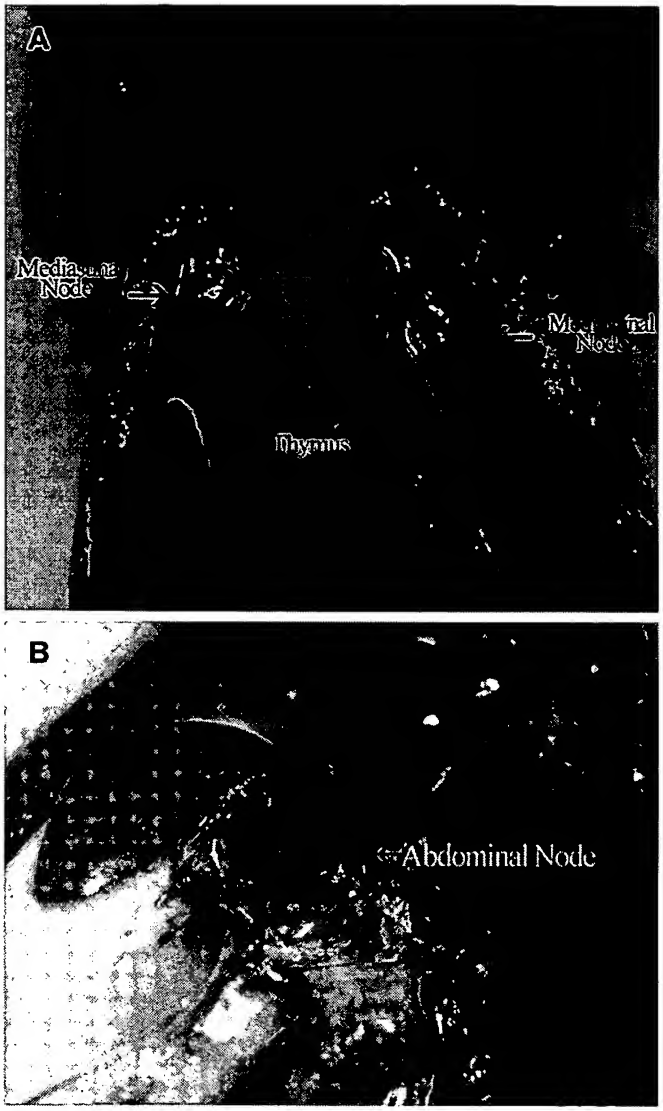

Fig. 2. A, photograph of mediastinal lymph nodes demonstrate the marked blue staining in experimental animals receiving avidin. Control animals had no apparent blue staining of these mediastinal nodes, even though they received the same intraperitoneal dose of ^{99m}Tc-blue-biotin liposomes. B, photograph of a blue stained abdominal lymph node in an experimental animal prior to dissection and removal. The control animal had no apparent staining of abdominal nodes.

intraperitoneally delivered liposomes, with the additional enhancements of greater prolongation of intraperitoneal retention and drug release as well as increased retention and release within lymph nodes.

In the last decade, research has been performed with intraperitoneally administered liposome-encapsulated anticancer agents (Malik et al., 1991; Vadiei et al., 1992; Daoud, 1994; Sharma et al., 1996, 1997). These studies have demonstrated a significantly prolonged retention time of the liposome-encapsulated chemotherapeutic agents in the peritoneum. These studies support the hypothesis that there is a marked pharmacological advantage for the treatment of intraperitoneal malignancies by encapsulating the intraperitoneally administered chemotherapeutic agent in a liposome (Vadiei et al., 1992; Daoud, 1994). Other studies demonstrate an improved toxicity profile. For instance, encapsulation of

paclitaxel in a liposome has been shown to have decreased toxicity while retaining equal efficacy for the treatment of intraperitoneal P388 leukemia (Sharma et al., 1996, 1997). It is likely that the reduced toxicity results from decreased local toxicity of encapsulated paclitaxel compared with the free drug. In humans, the dose-limiting toxicity from intraperitoneal administration of paclitaxel was severe abdominal pain, which was thought to be due to direct toxicity from either the paclitaxel or the ethanol/polyethoxylated castor oil delivery vehicle (Markman et al., 1992).

The observations from the present study suggest that the biotin-liposome/avidin methodology would enhance the reservoir-like effect observed previously for standard liposome formulations by blocking rapid lymphatic transit of liposomes from the peritoneum to the systemic circulation. The interaction of biotin-liposomes with avidin apparently results in aggregation of the liposomes in the peritoneum. This aggregation greatly alters the distribution of liposomes and seems to result in a prolonged retention of liposomes in the peritoneum as well as an increased accumulation and retention of liposomes in lymph nodes receiving drainage from the peritoneum. In our study, animals that received avidin had only a minimal percentage of the injected dose of liposomes reach the systemic circulation by 24 h as evidenced by the scintigraphic images and the low % ID found in the spleen, blood, and liver at 24 h (<9% ID). In contrast, control animals, not administered avidin, had 23% ID in the spleen, 14% ID in the blood, and 9.8% ID in the liver for a total of 47% ID in these organs at 24 h. Lymph nodes in the abdomen and in the mediastinum also had greatly increased uptake in the rats receiving avidin.

The liposome biodistribution for control animals in the present study was similar to previous reports with standard liposome formulations that were administered intraperitoneally (Ellens et al., 1981; Rosa and Clementi, 1983; Allen et al., 1993). For example, Ellens et al. (1981) reported that 19% of the intraperitoneally administered liposomes were detected in the blood at 2 h, with 7% in the liver and 4% in the spleen, indicating fairly rapid clearance of liposomes from the peritoneal cavity into the systemic circulation. In another study of intraperitoneally administered liposomes, 30% of the liposomes reached the liver by 6 h (Rosa and Clementi, 1983). Allen et al. (1993) has also demonstrated that liposomes labeled with iodine-125 and administered intraperitoneally to mice achieved peak blood levels that were very close to the situation when the same dose of liposomes were administered intravenously. After reaching the blood, the liposomes had a tissue distribution that was equal to that of intravenously injected liposomes.

It must be noted that simply making liposomes larger does not increase retention in the peritoneum or lymph nodes that receive drainage from the peritoneum. Hirono and Hunt (1985) have performed an extensive study on the effect of liposome size on their subsequent distribution after intraperitoneal administration. In their studies, 50 to 60% of the intraperitoneal dose of liposomes of varying sizes encapsulating ^{14}C-labeled sucrose cleared from the peritoneum by 5 h in all liposomes studied. These liposomes ranged in size from 48 to 720 nm. The greatest amount of [^{14}C]sucrose (~40%) appeared in the urine after administration of the largest liposomes. The authors speculated that the large 460- and 720-nm liposomes were unstable in the peritoneum so that they rapidly released the encapsulated [^{14}C]sucrose. It is also unlikely that simply increasing the size of the liposomes, in and of itself, would be sufficient to result in increased peritoneal and lymph node retention because particles as large as erythrocytes readily drain from the peritoneum by passing through the lymph nodes into the bloodstream. In one study of chromium-51-labeled red blood cells injected into the peritoneal cavity of sheep, 80% of the red cells had returned to circulation by 6 h after administration (Yuan et al., 1994).

In the present study, experimental animals retained a significant portion of the liposome dose in the abdominal region and in their abdominal and mediastinal nodes. This retention of liposomes in experimental animals should result in increased release of a liposome-encapsulated drug in the peritoneal fluid and in the lymph nodes receiving lymphatic drainage from the peritoneum. Delivery of liposome-encapsulated drugs using this method should provide sustained local release of drug within the peritoneum and the lymph nodes draining the peritoneum as the liposomes degrade or become phagocytized by macrophages. This delivery system could also attenuate systemic drug toxicities by greatly reducing the rate at which a drug returns to the systemic circulation by either passage through the lymphatic vessels and lymph nodes, or through direct absorption through the peritoneal membrane.

An important potential application of liposomes that encapsulate anticancer agents is in the prophylaxis of peritoneal carcinomatosis. Because 50% of patients with malignant gastrointestinal or gynecological diseases experience peritoneal carcinomatosis shortly after local curative resection, there is a great interest in delivering intraperitoneal chemotherapy during the perioperative period. In a recent study, Hribaschek et al. (2001) found that the intraperitoneal administration of the chemotherapeutic agents cisplatin and mitomycin prevented perioperative peritoneal carcinomatosis in a rat model. The rats receiving cisplatin did, however, experience severe, local toxicity with bleeding into the peritoneum and toxic necrotic reactions of the colon. Liposomes encapsulating anticancer agents delivered by the method described in this article could potentially be used for this type of perioperative chemotherapy. The potential for treatment of micrometastasis in lymph nodes secondary to lymphatic dissemination is also great. For example, liposome retention in mediastinal lymph nodes as demonstrated in this study may be efficacious in ovarian cancer therapy as metastasis to mediastinal and other lymph nodes are not uncommon findings in ovarian cancer at autopsy (Montero et al., 2000).

The investigations in this study were in normal animals. Future studies need to be conducted in models of various disease processes. Other investigations that need to be performed include determination the best timing of the avidin injection. This timing may vary depending on the disease process being treated. For example, administration of the avidin simultaneously with the liposomes is likely to result in a significantly greater retention in the peritoneum. Other variations in timing could result in increased lymph node or diaphragm targeting of the liposomes. One significant consideration would be whether this method would have any significant advantage in an advanced disease process if the lymphatics were obstructed. With lymphatic obstruction, intraperitoneally administered liposomes would likely be retained in the peritoneum and would be unable to reach the

lymph nodes so that the advantages of this delivery system would be negated. In most intraperitoneal disease processes, complete obstruction of the lymphatics is uncommon and generally occurs only in advanced disease. For example, ovarian cancer is the most common cause of malignant ascites, which is often due to lymphatic obstruction. However, in a recent study only 12.5% of women diagnosed with early stage ovarian cancer presented with ascites (Eltabbakh et al., 1999).

In summary, the intraperitoneal biotin-liposome/avidin delivery method described in this article has potential as a delivery system for the local treatment of intraperitoneal and intralymphatic disease processes by increasing the retention of drugs in the peritoneum and in the lymph nodes that receive lymphatic drainage from the peritoneum.

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Avidin/Biotin-Liposome System Injected in the Pleural Space for Drug Delivery to Mediastinal Lymph Nodes

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ABSTRACT: The objective of this study was to develop a more effective liposome-based method for delivering drugs to mediastinal nodes. Nodal uptake was determined after intrapleural injection of the avidin/biotin-liposome system in normal rats. The effect of injection sequence (avidin injected 2 h before biotin-liposomes and vice versa), volume injected, and administered dose of the agents is described. Pharmacokinetics of the avidin/biotin-liposome system was monitored with scintigraphic imaging by labeling the biotin-liposomes with technetium-99m (^{99m}Tc). To identify the nodes during the biodistribution studies, patent blue dye was encapsulated in the biotin-liposomes. Tissue biodistribution studies were performed 22 h after injection of the ^{99m}Tc-blue-biotin-liposomes. When avidin was injected before ^{99m}Tc-blue-biotin-liposomes, better mediastinal node targeting (15.7%; $p < 0.05$) was achieved than when biotin-liposomes were injected first (8.3%) or when only biotin-liposomes were injected (1.0%). Injection of a small dose of liposomes (0.5 mg phospholipid) and avidin (0.5 mg) resulted in the most favorable drug delivery to mediastinal nodes and other organs. Intrapleural injection of the avidin/biotin-liposome system could potentially be used for drug delivery to disease processes such as lung cancer, anthrax, and tuberculosis that invade mediastinal nodes and use them as centers of incubation and dissemination. J Pharm Sci 93:2595–2608, 2004

Keywords: liposomes; drug targeting; pharmacokinetics; lymphatic transport; scintigraphy

INTRODUCTION

Lymphatic spread is one of the common pathways that cancer and other diseases use to disseminate.[1,2] Tumor cells, viruses, and bacteria use the lymphatics to spread and form secondary tumors and sites of infection. The lymphatic system is a complex network of channels that drain fluids and proteins from the interstitial space of the body and transport them to the lymph nodes and then into the blood circulation. Important functions of the lymph nodes include defending the body against infections by filtering invading particles from the lymph fluid and supporting the activities of the immune system.[3,4] However, if the system is severely affected or invaded by a disease, the lymph nodes can act as holding reservoirs where these agents can take root and expand into other regions of the body.[5,6] In consequence, important attention has been focused on the development of effective methods to deliver therapeutic agents to lymph nodes to prevent further dissemination of the disease.[5,7–9]

One approach to this problem is the use of liposomes to carry drugs to the lymphatic system with the goal of improving lymph node targeting while reducing toxicity and adverse side reactions caused by the "free" drug. Liposomes offer several advantages over other delivery systems including biocompatibility, protection of the drug from degradation, and a wide range of physical properties

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that can be modified to control their biological behavior.[10,11] Targeting of lymph nodes with liposomes after subcutaneous and intraperitoneal administration has been previously reported.[12–18]

In the present work, we propose the use of the avidin/biotin-liposome system for the targeting of mediastinal lymph nodes. Mediastinal lymph nodes are involved in many pathological processes including lung cancer, tuberculosis, and anthrax,[19–23] and represent a critical site for cancer and infection spread into other regions of the body. There are few reports describing mediastinal node targeting and only intraperitoneal injection of certain liposome formulations has shown promising results.[12,18]

The avidin/biotin-liposome system consists of avidin and biotin-coated liposomes, and makes use of the high affinity between avidin and biotin.[24] As previously described, the interaction of both agents during their migration into the lymphatic vessels can produce an aggregate of liposomes that is better retained in lymph nodes when injected subcutaneously and intraperitoneally.[17,18] Now, we propose injecting the avidin/biotin-liposome system directly into the pleural space to target mediastinal nodes. In a previous work,[25] we described the pharmacokinetics and biodistribution of indium-111-avidin and technetium-99m (^{99m}Tc) biotin-liposomes injected individually in the pleural space and peritoneum. Comparing intraperitoneal versus intrapleural injections of each individual agent, the results showed that intrapleural injection offers some advantages in the targeting of mediastinal nodes, in particular a significant reduction of unfavorable distribution of the agents in spleen, kidney, and bowel.[25] This study also revealed that a single injection of avidin had better lymphatic uptake and mediastinal node retention than a separate single injection of biotin-liposomes. This result led us to hypothesize that an aggregate of liposomes could be produced and retained in the nodes if avidin was injected first and then followed some time later by an injection of biotin-liposomes.

In the present work, we investigated the potential of intrapleural injection of the avidin/biotin-liposome system for delivering liposome-encapsulated drugs to mediastinal nodes. The effect of the sequence of the intrapleural injection of avidin and the ^{99m}Tc-blue-biotin-liposomes as well as the volume in which they were injected was investigated in normal rats. An injection of avidin followed 2 h later with an injection of biotin-liposomes (Method A) was compared with an injection of biotin-liposomes followed 2 h later by an injection of avidin (Method B), and with a control group (Method C) in which no avidin was injected (Fig. 1). In each method, three different volumes of both agents were injected. In a second experiment, mediastinal node targeting was evaluated by comparing different injected doses of both agents.

The pharmacokinetics and organ uptake of the avidin/biotin-liposome system was monitored with a gamma camera by labeling the biotin-liposomes with ^{99m}Tc. In order to visualize the nodes during the biodistribution studies, patent blue dye was encapsulated in the biotin-liposomes. Tissue biodistribution studies were performed 22 h after injection of the labeled liposomes.

EXPERIMENTAL

Blue Biotin-Liposome Manufacture and Characterization

Liposomes coated with biotin were comprised of distearoyl phosphatidylcholine (Avanti Polar

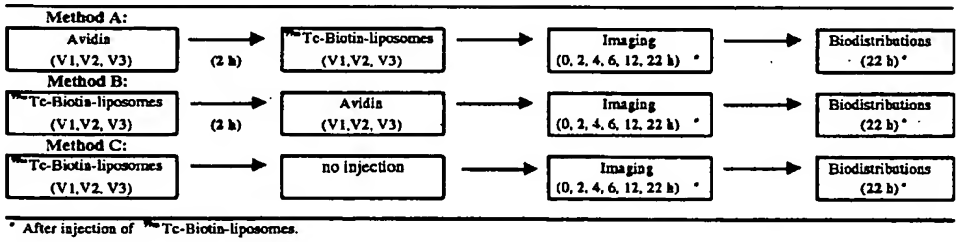

Figure 1. Experimental design for avidin and ^{99m}Tc-blue-biotin-liposome administration at different volumes (V1: 0.05 mL of ^{99m}Tc-biotin-liposomes, 0.03 mL of avidin; V2: 0.1 mL of ^{99m}Tc-biotin-liposomes, 0.06 mL of avidin; V3: 0.5 mL of ^{99m}Tc-biotin-liposomes, 0.3 mL of avidin) in the pleural space. The sequence of the injections, image acquisition, and biodistributions for each method tested are shown. Same dose, D1: 0.5 mg of phospholipid (1.73 mg/kg-body) and 0.5 mg of avidin was used in these studies.

Lipids, Pelham, AL)/cholesterol (Calbiochem, San Diego, CA)/*N*-biotinoyl distearoyl phosphoethanolamine (Northern Lipids, Vancouver, Canada)/α-tocopherol (Aldrich, Milwaukee, WI) (58:39:1:2 total lipid molar percentage). Lipid ingredients were co-dried from chloroform (Fisher Scientific, Fair Lawn, NJ) by removing it by rotary evaporation and desiccation for 24 h. The dried lipid film was rehydrated with 300 mM sucrose (Sigma, St. Louis, MO) in sterile water (at a total lipid concentration of 120 μmol/mL), warmed to 55°C, and then lyophilized overnight. The dried lipid-sucrose mixture was rehydrated with 200 mM reduced glutathione (GSH) (Sigma) in Dulbecco's phosphate-buffered saline (PBS) (pH 6.3) and 10 mg/mL patent blue dye (CI 42045; Sigma). Before extrusion, the lipid suspension was diluted to 40 μmol/mL with 100 mM GSH in PBS containing 150 mM sucrose and 10 mg/mL patent blue dye and then extruded through a series (2 μ, two passes; 400 nm, two passes; 100 nm, five passes) of polycarbonate filters (Lipex Extruder, Vancouver, Canada) at 55°C to form unilamellar liposomes. Unencapsulated GSH, blue dye, and sucrose were then removed by repeated washings and ultracentrifugation at 41,000 rpm for 50 min (Ti50.2 rotor; Beckman, Fullerton, CA). Liposomal pellets were resuspended in PBS containing 300 mM sucrose (pH 6.3) to a total lipid concentration of 60 μmol/mL and stored at 4°C until needed.

Liposome preparations were tested for liposomal size by determining the diameter and polydispersity index (PI) using a particle size analyzer (Brookhaven Instruments, Holtsville, NY). The PI measures the homogeneity of the liposome sample with regard to diameter. A value of 0.0 represents a completely homogeneous sample whereas a value of 1.0 represents a completely heterogeneous sample. Liposomes were also tested in duplicate for phospholipid content by Stewart assay[26]; GSH content by BIOXYTECH GSH-400 assay (R&D Systems, Minneapolis MN); blue dye concentration by spectrophotometry at 635 nm; sterility and endotoxin levels. After release from liposomes by chloroform extraction, GSH was separated from blue dye using a D-salt Polyacrylamide 1800 Desalting Gel column (Pierce, Rockford, IL) before determination of GSH content with GSH-400 assay.

Liposome Labeling Procedure

The labeling of liposomes with ^{99m}Tc was performed as previously described.[27] Briefly, a commercial kit of lipophilic chelator, hexamethylpropyleneamine oxime (HMPAO, Ceretec; Nycomed Amersham, Arlington Heights, IL), was reconstituted with either 5 mL of saline containing 555 MBq (15 mCi) or 2.5 mL containing 2775 MBq (75 mCi) of ^{99m}Tc-pertechnetate (Nycomed Amersham Radiopharmacy, San Antonio, TX) depending on the final specific activity of the liposomes needed for small-volume injections. An aliquot of ^{99m}Tc-HMPAO was added to a concentrated suspension of blue biotin-liposomes (1:1 ratio) encapsulating GSH and incubated at room temperature for 30 min. Free label was removed from the liposomes by passage over a Sephadex G-25 column (Amersham Pharmacia Biotech; Uppsala, Sweden). Labeling efficiencies were checked by determining the ^{99m}Tc activity before and after column separation of ^{99m}Tc-blue-biotin-liposomes using a dose calibrator (Mark 5; Radix, Houston, TX).

Animal Studies

Male Sprague-Dawley rats (~0.3 kg) were used for these experiments. Animal experiments were performed under the National Institutes of Health Animal Use and Care Guidelines and approved by our institutional animal committee. Before injections, imaging, and euthanasia, the rats were anesthetized by inhalation with isoflurane-USP (Vedco, Inc., St. Joseph, MO) using an anesthesia inhalation machine (Backford Inc., Wales Center, NY).

Intrapleural Injection Technique

Injections in the pleural space were performed using the following technique. Anesthetized rats were shaved in the lateral left chest. An incision of approximately 8 mm was made through the skin, then the fascia was dissected away and a small incision was made through the external oblique muscle layer, the latissimus dorsi, and the serratus layers. Using fine scissors, a nick was made in the intercostal layers. The intercostal layers were punctured using a blunt needle stub (19 gauge, ~4.5 mm in length). To confirm penetration and to prevent damage to the underlying lungs, a 1-mL tuberculin syringe was fitted to the 19-gauge luer hub and 0.1 mL of air was injected into the pleural space. When successfully placed, the air will enter the pleural space without resistance. If resistance was encountered, the 19-gauge stub was removed and reintroduced. The

material was then injected using a blunt 23-gauge needle stub (~20 mm in length) inserted through the 19-gauge needle stub.

Study Design

Pilot studies were performed to evaluate the optimal time to intrapleurally inject the avidin and biotin-liposomes based on pharmacokinetics results obtained in a previous study.[25] When both agents were injected at the same time, avidin followed immediately by the biotin-liposomes, a large liposome aggregate was largely confined in the pleural space and the targeting of mediastinal nodes was minimal (<1%). Increasing the time between injections to 30 min also resulted in large levels of liposome aggregate confined in the pleural space and low mediastinal node targeting. When the time between injections was increased to 4 h, minimal liposome aggregate was produced with the biotin-liposomes moving quickly into blood circulation and spleen and minimal retention in mediastinal nodes. When the time between injections was fixed at 2 h, liposome aggregate was poorly confined in the pleural space and high levels of mediastinal node targeting were achieved. In the following experiments, a time of 2 h between the injections of the agents was used.

Injection Sequence and Injected Volume Study

Mediastinal node targeting as a function of the injection sequence of each agent and the volume injected was determined. In the first injection sequence (Method A—avidin first), avidin (Sigma) was injected in the pleural space followed 2 h later by an injection of ^{99m}Tc-blue-biotin-liposomes injected in the same injection site. In the second injection sequence (Method B—biotin-liposomes first), ^{99m}Tc-blue-biotin-liposomes were injected first and the avidin was injected 2 h later. For the control group (Method C—control), only ^{99m}Tc-blue-biotin-liposomes were injected in pleural space (Fig. 1). For each method, three different volumes (V1, V2, V3) of each agent were injected maintaining a constant dose D1 of phospholipid and avidin. Clinical experience with pleural diseases and experimental results in animals[28,29] have suggested that the lymphatic clearance of the pleural space operates at maximum rate once the pleural liquid exceeds a certain threshold volume. In most mammals, the volume of pleural liquid in normal conditions is 0.1–0.2 mL/kg.[30] According with this value, the volume of pleural liquid in rats (300 g) should be 30–60 μL. In this experiment, three different volumes were injected to study the effect of the injected volume (≥the threshold volume) on the targeting and retention of liposomes in mediastinal nodes. The injected dose D1 (0.5 mg of phospholipid and 0.5 mg of avidin) was empirically determined from a previous lymph node targeting study in which avidin and biotin-liposomes were subcutaneously injected.[17] For the first volume, V1, 0.05 mL of ^{99m}Tc-blue-biotin-liposomes (0.52 mg ≈ 1.73 mg of phospholipid/kg-body; 12 MBq) and 0.03 mL of avidin solution in saline (0.5 mg) were used. For V2, 0.1 mL of ^{99m}Tc-blue-biotin-liposomes (0.52 mg ≈ 1.73 mg of phospholipid/kg-body; 9.4 MBq) and 0.06 mL of avidin (0.5 mg) were injected. For V3, 0.5 mL of ^{99m}Tc-blue-biotin-liposomes (0.52 mg ≈ 1.73 mg of phospholipid/kg; 9.0 MBq) and 0.3 mL of avidin (0.5 mg) were used.

Injected Dose Study

To evaluate mediastinal node targeting as a function of the injected dose of both agents, three different doses were injected in the pleural space using the procedure of Method A-V2 because these parameters resulted in the best mediastinal node targeting as determined from the prior injection sequence and volume injected study (Table 1). Three groups of rats were used for this study. In the first group, D1, a dose of 0.5 mg/0.06 mL of avidin and 0.5 mg/0.1 mL of phospholipid (^{99m}Tc-blue-biotin-liposomes; ~1.67 mg of phospholipid/kg-body; 9.4 MBq) was injected. For the second group, D2, 1.0 mg/0.06 mL of avidin and 1.0 mg/0.1 mL of phospholipid (~3.34 mg of phospholipid/kg-body, 23 MBq) were injected. Finally, in the last group, D3, 5.0 mg/0.06 mL of avidin and 5.0 mg/0.1 mL of phospholipid (~16.7 mg of phospholipid/kg-body, 17.8 MBq) were used.

Image Acquisition

In all these studies, scintigraphic images were acquired at several times. Static images were acquired in a 128 × 128 Word Image Matrix with a zoom of 1.45× using a Picker SX-300 gamma camera (Cleveland, OH) interfaced to a Medasys Pinnacle computer (Miami, FL). Images for the ^{99m}Tc-blue-biotin-liposomes were acquired in the ^{99m}Tc window (140 keV ± 20%) using a low-energy high-resolution collimator.

Table 1. Biodistribution (% ID/Organ) of ^{99m}Tc-Blue-Biotin-Liposomes in the Presence and Absence of Avidin at 22 h after Pleural Injection in Rats

	% ID/organ								
	Method A			Method B			Method C		
Organ	V1 ($n=5$)	V2 ($n=4$)	V3 ($n=4$)	V1 ($n=7$)	V2 ($n=4$)	V3 ($n=4$)	V1 ($n=4$)	V2 ($n=3$)	V3 ($n=3$)
Blood	0.3 ± 0.04	0.03 ± 0.02	0.2 ± 0.01	0.9 ± 0.4	0.4 ± 0.1	0.5 ± 0.2	$12.1\pm1.4^{g,h}$	$13.8\pm1.9^{g,h}$	$7.4\pm2.1^{g,h}$
Heart	0.1 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.1 ± 0.01	0.06 ± 0.01	0.1 ± 0.08	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.01
Lung	0.8 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.2	0.6 ± 0.1	0.5 ± 0.05	0.8 ± 0.09	0.5 ± 0.08
Thymus	0.5 ± 0.1	0.3 ± 0.02	0.3 ± 0.04	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.06	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.09
Mediastinal nodes	11.3 ± 1.1	15.7 ± 1.4	7.2 ± 0.6	8.3 ± 1.6^{b}	$4.9\pm0.7^{a,b}$	$5.2\pm1.2^{a,b}$	$0.8\pm0.1^{g,h}$	$1.0\pm0.1^{g,h}$	$0.9\pm0.1^{g,h}$
Liver	6.4 ± 1.3	8.8 ± 1.2	4.7 ± 1.4	8.2 ± 1.0	9.4 ± 0.9	6.8 ± 1.5	11 ± 1.5^{c}	13.5 ± 0.4^{c}	12.8 ± 1.0^{c}
Spleen	0.4 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	2.3 ± 0.3^{g}	3.2 ± 0.3^{g}	1.7 ± 0.4^{g}	$9.7\pm0.6^{g,h}$	$11.7\pm0.8^{g,h}$	$9.9\pm0.6^{g,h}$
Kidney	2.7 ± 0.3	2.8 ± 0.1	2.5 ± 0.2	2.6 ± 0.2	3.7 ± 0.2^{d}	2.4 ± 0.1^{e}	$4.2\pm0.5^{d,f,g}$	$5.0\pm0.1^{d,f,b}$	$4.3\pm0.2^{d,f,g}$
Diaphragm	1.0 ± 0.2	0.7 ± 0.1	1.3 ± 0.6	0.9 ± 0.3	0.8 ± 0.1	0.5 ± 0.04	0.3 ± 0.02	0.7 ± 0.05	0.9 ± 0.2
Pericardium	11.2 ± 1.9	11.0 ± 1.8	8.4 ± 1.9	4.7 ± 0.8	5.7 ± 1.0	11.7 ± 2.4	$1.5\pm0.2^{f,g}$	$3.3\pm1.4^{a,b,c,f}$	$2.2\pm0.2^{f,g}$
Urine	19.7 ± 3.2	18.7 ± 0.7	20.0 ± 0.7	18.1 ± 2.0	22.1 ± 0.8	20.3 ± 0.4	13 ± 2.6	22.4 ± 0.6	22.3 ± 4.6
Feces	3.3 ± 1.9	1.6 ± 0.6	0.7 ± 0.2	2.8 ± 0.7	1.3 ± 0.1	0.7 ± 0.3	3.2 ± 0.5	7.2 ± 0.6	4.7 ± 0.5
Bowel	4.2 ± 1.1	3.6 ± 1.0	4.2 ± 0.2	3.4 ± 0.5	7.01 ± 0.5	6.5 ± 0.4	9.7 ± 1.3	1.6 ± 0.5	6.2 ± 1.4

Values are the mean ± SEM. In each volume, the same dose (D1 = 0.5 mg) of avidin and phospholipid was injected. Method A: avidin followed 2 h later by ^{99m}Tc-blue-biotin-liposomes; Method B: ^{99m}Tc-blue-biotin-liposomes followed 2 h later by avidin; Method C: ^{99m}Tc-blue-biotin-liposomes (control group); V1: 0.05 mL ^{99m}Tc-blue-biotin-liposomes and 0.03 mL avidin; V2: 0.1 mL ^{99m}Tc-blue-biotin-liposomes and 0.06 mL avidin; V3: 0.5 mL ^{99m}Tc-blue-biotin-liposomes and 0.3 mL avidin.

$p < 0.05$, one way ANOVA. [a]vs. Method A-V1; [b]vs. Method A-V2; [c]vs. Method A-V3; [d]vs. Method B-V1; [e]vs. Method B-V2; [f]vs. Method B-V3; [g]vs. Method A (all volumes); [h]vs. Method B (all volumes).

Biodistributions

Biodistribution studies were performed 22 h after ^{99m}Tc-blue-biotin-liposome injection. After imaging, the anesthetized animals were euthanized by cervical dislocation, and tissues were harvested, weighed, and counted for radioactivity (Auto-Gamma 5000 Series; Packard Instruments, Downers Grove, IL). Total blood volume was calculated as 5.6% of total body weight.[31] During the entire study, the rats were located in individual metabolic cages and the urine and feces were collected. Feces and bowel were dissolved in a saturated solution of sodium hydroxide (NaOH). An aliquot of the total volume of urine and the dissolved feces and bowel were counted for radioactivity. The percentage of injected dose (% ID) per organ was calculated by comparison with a standard aliquot of the radioactive material used.

Image Analysis

Regions of interest (ROI) were drawn around the mediastinal nodes and selected organs on the images using OSIRIS imaging software (version 3.1; University Hospital of Geneva, Geneva, Switzerland). A box was drawn around the entire body in the baseline image (0 h) to determine the number of counts injected into the body (A_0). The percentage of activity at some specific time t [$\%A(t)$] accumulated in the ROI was calculated as the ratio of the counts in the ROI [$A_{ROI}(t)$] and the total number of counts injected into the whole body at baseline (A_0), multiplied by 100 [$\%A(t) = A_{ROI}(t)/A_0 \times 100$]. Counts associated with the ROI were corrected for radioactive decay (referred to the injection time), the time used for image acquisition, and for background activity when the time of image acquisition was longer than 15 min.

Statistical Analysis

Values are reported as mean ± SEM. Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA) to compare the percentage of injected dose per organ (% ID/organ) in the mediastinal nodes and other organs using SPSS Base 10.0 software (SPSS Inc., Chicago, IL). Rats were considered nested between method and volume or between dose. Differences in % ID/organ were statistically compared using between-groups multiple comparisons. When necessary, comparison among means were Bonferroni adjusted. A log transformation was applied to the data to better satisfy the assumptions underlying the analysis. Means and standard errors were computed from untransformed data and statements of statistical significance were based on transformed data. $p < 0.05$ was defined as an acceptable probability for a significant difference between means.

RESULTS

Liposome Characterization

Liposomes were 134 nm (0.079 PI) in diameter and contained 37 mmol/L phospholipid and 0.046 mmol/L GSH. No growth of bacteria was detected and the endotoxin level was >2.5 EU/mL and <5 EU/mL. Concentration of blue dye was 0.836 mg/mL. The labeling efficiency was between 80 to 90% in all these experiments.

Mediastinal Node Targeting as a Function of the Injection Sequence and Volume Injected

Image Analysis

Figure 2 shows the 22-h whole-body scintigraphic images depicting ^{99m}Tc-blue-biotin-liposome activity distribution and targeting in mediastinal nodes. For each Method A–C, an image is presented for the three different injection volumes, V1–V3. Figure 2, A and B show the images when Method A and Method B were used, respectively. Figure 2C shows the control group depicting the ^{99m}Tc-blue-biotin-liposome distribution in the absence of avidin. As compared with the control group, a better retention of ^{99m}Tc-blue-biotin-liposomes in mediastinal nodes was observed with either Method A or Method B. The images in Figure 2C indicate that ^{99m}Tc-blue-biotin-liposomes in the absence of avidin, leave the pleural space and move into blood circulation, and then are removed from the blood by the spleen and liver. A different situation is observed when the avidin/biotin-liposome system is used. In the presence of avidin, blood and spleen accumulation of liposomes is greatly reduced.

Figure 3 depicts the kinetic behavior of the ^{99m}Tc-blue-biotin-liposomes based on the image analysis of the different studies. In Figure 3A, the percentage of activity detected in the thoracic cavity is depicted as a function of time. Independent of the injection volume, when Method A was used, the rate of liposome removal was slow (<30%

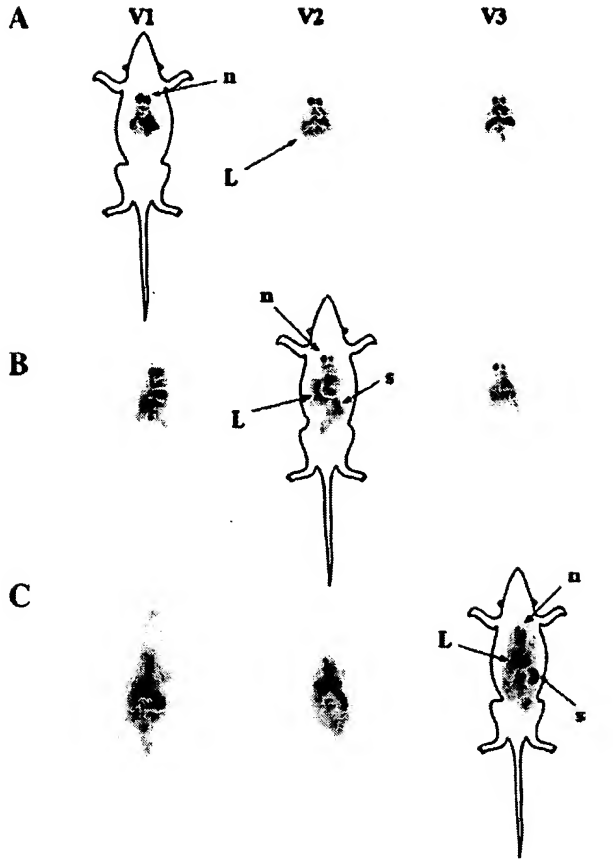

Figure 2. Scintigraphic images depicting the 22-h activity distribution of ^{99m}Tc-blue-biotin-liposomes after injection of the avidin/biotin-liposome system in the pleural space. For each Method A–C, an image is presented for the three different injection volumes, V1–V3 with the dose D1. V1: 0.05 mL of ^{99m}Tc-biotin-liposomes, 0.03 mL of avidin; V2: 0.1 mL of ^{99m}Tc-biotin-liposomes, 0.06 mL of avidin; V3: 0.5 mL of ^{99m}Tc-biotin-liposomes, 0.3 mL of avidin; D1: 0.5 mg of phospholipid (1.73 mg/kg-body) and 0.5 mg of avidin. L, liver; s, spleen; and n, mediastinal nodes.

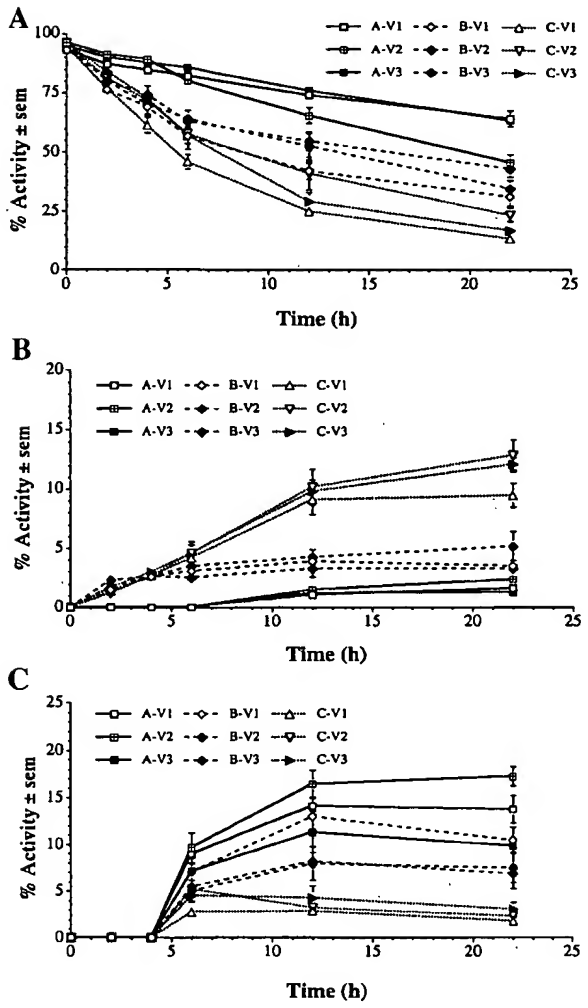

Figure 3. Image analysis results depicting the kinetics of ^{99m}Tc-blue-biotin-liposomes in terms of the percentage of activity in (A) thoracic cavity, (B) spleen, and (C) mediastinal nodes, after injection in the pleural space of the avidin/biotin-liposome system using Method A-V1–V3, Method B-V1–V3, and Method C-V1–V3 ($n = 3$–7 animals/group). Same dose D1 [0.5 mg of phospholipid (1.73 mg/kg-body) and 0.5 mg of avidin] was used in each case. V1: 0.05 mL of ^{99m}Tc-biotin-liposomes, 0.03 mL of avidin; V2: 0.1 mL of ^{99m}Tc-biotin-liposomes, 0.06 mL of avidin; V3: 0.5 mL of ^{99m}Tc-biotin-liposomes, 0.3 mL of avidin; D1: 0.5 mg of phospholipid (1.73 mg/kg-body) and 0.5 mg of avidin.

had left the thoracic region at 12 h) as compared with Methods B and C in which between 50 to 75% of the ^{99m}Tc-blue-biotin-liposomes had already moved from the thoracic cavity by 12 h.

This behavior is evident in Figure 3B depicting the percentage of activity in spleen. In the absence of avidin, ^{99m}Tc-blue-biotin-liposomes moved quickly from the pleural space into blood circulation via the thoracic lymphatics and then into the organs of the reticuloendothelial system, principally the spleen. By 12 h, liposome accumulation in spleen was ~10% for the Method C groups, ~4% for the Method B groups, and <2% for Method A groups. It is important to mention that during the first 6 h, minimal activity was detected in spleen when Method A was used. Also, during the first 6 h, no difference in the kinetics of liposomes between Method B and Method C was detected. However, after 6 h, liposomes in the Method B groups began to move slower. The same situation was observed in the thoracic cavity (Fig. 3A).

Figure 3C depicts the percentage of activity in mediastinal nodes. No results of percentage of activity are presented during the first 4 h because it is hard to differentiate between activity in the nodes and the overlaying activity in the thoracic cavity in the images. At 6 h, nodal accumulation was very similar between Methods B and C, but after 6 h, it was observed that liposomes injected by Method C were just passing through the nodes and moving into blood circulation. When avidin was injected, either before or after biotin-liposome injection, retention of liposomes in nodes was evident. At 12 and 22 h, differences in the retention of liposomes in nodes between Methods A and B were more easily distinguished. The results indicated Method A > Method B > Method C in terms of mediastinal node targeting. Also, injection of small volumes (V1 and V2) showed the best result in terms of mediastinal node targeting by Method A.

Biodistribution Results

Tables 1 and 2 show the 22-h biodistribution results in terms of the percentage of injected dose per organ and percentage of injected dose per gram of tissue as a function of the injection sequence and the injected volume. From the results, it is evident that the avidin/biotin-liposome system, independent of the volume injected in the pleural space, reduces entrance of liposomes into the blood circulation as compared with the control group (Method C) where no avidin was injected ($p < 0.05$). In terms of mediastinal node targeting, the avidin/biotin-liposome system injected either by Method A or Method B showed much better retention in the nodes than the control group.

One-way ANOVA (between the nine groups) for mediastinal node accumulation indicated that groups A-V1 and A-V2 were statistically different ($p < 0.05$) from the other groups B-V1 to C-V3, but not different from A-V3 or between each other. Also, an interaction-effect between volume and method was found when a two-way ANOVA was performed ($p = 0.03$). A two-way ANOVA *post hoc* test showed that group A-V2 resulted in the best targeting of liposomes, as compared with the other groups ($p < 0.05$). For the case of other important organs (spleen and kidney), the results showed low levels of accumulation when the avidin/biotin-liposome system was used (see Table 1). An important result was observed with spleen, where independent of the volume injected, Method A resulted in the lowest retention of liposomes in that organ ($p < 0.05$).

Injected Dose Study

Biodistribution Results

Table 3 shows the 22-h biodistribution results when Method A-V2 was used to evaluate mediastinal node targeting as a function of the dose injected. The smallest injected dose, D1 (0.5 mg of avidin and 0.5 mg of phospholipid), resulted in the best targeting of mediastinal nodes (15.7%; $p < 0.05$). No statistical difference in accumulation of liposomes in blood, spleen, and kidney was observed between D1 and D2 (1.0 mg of avidin and 1.0 mg of phospholipid). For the same organs, D3 (5.0 mg of avidin and 5.0 mg of phospholipid) resulted in the highest accumulation of liposomes ($p < 0.05$). However, no difference in liposome accumulation in liver was detected between any of the groups.

Figure 4 shows the ratio of the % ID/g of ^{99m}Tc-blue-biotin-liposomes in mediastinal nodes and in other major organs for the injected doses, D1–D3. When dose D1 was injected, 51,500-fold more liposomes accumulated in lymph nodes as compared with blood, >638-fold as compared with liver, >608-fold as compared with spleen, and >414-fold as compared with kidney. In contrast, when dose D2 was injected, 29,300-fold more liposomes accumulated in lymph nodes as compared with blood, >522-fold as compared with liver, 436-fold as compared with spleen, and 273-fold as compared with kidney. In all these cases, D1 ratios were always higher than D2 ratios (Fig. 4). It was observed that the larger dose D3 resulted in the lowest ratio values as compared with the other doses with only 1417-fold more liposomes in lymph nodes compared with blood, >221-fold as compared with liver, 37-fold compared with spleen, and 225-fold compared with kidney.

Blue Dye Delivered in Mediastinal Nodes

As an example of the potential of the avidin/biotin-liposome system for the delivery of drugs in mediastinal nodes, Table 4 shows the amount of blue dye delivered by liposomes in mediastinal nodes and in other major organs. Using the assumption that the total amount of blue dye encapsulated in liposomes is proportional to the phospholipid dose injected, the amount of blue dye transported in liposomes was calculated to

Table 2. Biodistribution (% ID/g) of ^{99m}Tc-Blue-Biotin-Liposomes in the Presence and Absence of Avidin at 22 h after Pleural Injection in Rats

	% ID/g								
	Method A			Method B			Method C		
Organ	V1 ($n=5$)	V2 ($n=4$)	V3 ($n=4$)	V1 ($n=7$)	V2 ($n=4$)	V3 ($n=4$)	V1 ($n=4$)	V2 ($n=3$)	V3 ($n=3$)
Blood	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.1 ± 0.0	0.02 ± 0.01	0.03 ± 0.01	$0.7 \pm 0.1^{g,h}$	$0.8 \pm 0.1^{g,h}$	$0.4 \pm 0.2^{g,h}$
Heart	0.1 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.1 ± 0.01	0.06 ± 0.01	0.1 ± 0.08	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.01
Lung	0.8 ± 0.2	0.7 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.9 ± 0.2	0.6 ± 0.1	0.5 ± 0.05	0.8 ± 0.09	0.5 ± 0.08
Thymus	1.4 ± 0.23	0.7 ± 0.04	0.8 ± 0.14	1.2 ± 0.3	1.12 ± 0.3	1.1 ± 0.2	1.1 ± 0.2	1.7 ± 0.4	1.5 ± 0.5
Mediastinal nodes	394 ± 75	515 ± 34	263 ± 32	353 ± 63^{b}	$270 \pm 64^{a,b}$	$223 \pm 47^{a,b}$	$32 \pm 5^{g,h}$	$36 \pm 4^{g,h}$	$26 \pm 6^{g,h}$
Liver	0.64 ± 0.13	0.84 ± 0.1	0.4 ± 0.13	0.9 ± 0.1	1.1 ± 0.1	0.7 ± 0.15	1.1 ± 0.2^{c}	1.4 ± 0.1^{c}	1.2 ± 0.1^{c}
Spleen	0.7 ± 0.11	0.9 ± 0.1	0.6 ± 0.1	4.1 ± 0.5^{g}	5.0 ± 0.7^{g}	2.5 ± 0.7^{g}	$18.6 \pm 1.3^{g,h}$	$17.0 \pm 2.1^{g,h}$	$14.2 \pm 2.2^{g,h}$
Kidney	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	1.9 ± 0.2^{d}	1.2 ± 0.1^{e}	$2.5 \pm 0.2^{d,f,g}$	$2.4 \pm 0.1^{d,f,b}$	$2.1 \pm 0.1^{d,f,g}$
Diaphragm	1.5 ± 0.3	0.9 ± 0.2	1.5 ± 0.7	1.6 ± 0.5	1.0 ± 0.1	0.7 ± 0.05	0.4 ± 0.04	0.9 ± 0.06	1.1 ± 0.2
Pericardium	141 ± 21	125 ± 4.0	79 ± 28	106 ± 14	121 ± 27	128 ± 23	$22 \pm 1.5^{f,g}$	$41 \pm 15^{a,b,c,f}$	$35 \pm 16^{f,g}$

Values are the mean ± SEM. In each volume, the same dose (D1 = 0.5 mg) of avidin and phospholipid was injected. Method A: avidin followed 2 h later by ^{99m}Tc-biotin-liposomes; Method B: ^{99m}Tc-biotin-liposomes followed 2 h later by avidin; Method C: ^{99m}Tc-biotin-liposomes (control group); V1: 0.05 mL ^{99m}Tc-biotin-liposomes and 0.03 mL avidin; V2: 0.1 mL ^{99m}Tc-biotin-liposomes and 0.06 mL avidin; V3: 0.5 mL ^{99m}Tc-biotin-liposomes and 0.3 mL avidin.

$p < 0.05$, one way ANOVA. [a]vs. Method A-V1; [b]vs. Method A-V2; [c]vs. Method A-V3; [d]vs. Method B-V1; [e]vs. Method B-V2; [f]vs. Method B-V3; [g]vs. Method A (all volumes); [h]vs. Method B (all volumes).

Table 3. Effect of Injected Dose of Avidin/Biotin-Liposome System on the Distribution of ^{99m}Tc-Blue Biotin-Liposomes at 22 h Post-Injection

	% ID/organ			% ID/g		
Organ	D1 ($n=4$)	D2 ($n=7$)	D3 ($n=3$)	D1 ($n=4$)	D2 ($n=7$)	D3 ($n=3$)
Blood	0.3 ± 0.02	0.2 ± 0.02	3.0 ± 1.23[a,b]	0.01 ± 0.001	0.01 ± 0.00	0.15 ± 0.06[a,b]
Heart	0.05 ± 0.005	0.1 ± 0.01	0.2 ± 0.03	0.04 ± 0.01	0.1 ± 0.02	0.2 ± 0.03
Lung	0.80 ± 0.19	0.7 ± 0.2	0.7 ± 0.03	0.7 ± 0.2	0.7 ± 0.2	0.6 ± 0.03
Thymus	0.3 ± 0.02	0.4 ± 0.15	0.2 ± 0.03	0.7 ± 0.04	1.1 ± 0.14	0.7 ± 0.13
Mediastinal nodes	15.7 ± 1.39	8.8 ± 1.21[a]	6.9 ± 0.42[a]	515.3 ± 33.4	277.7 ± 40.3	154.9 ± 16.3[a]
Liver	8.8 ± 1.24	6.0 ± 1.37	8.2 ± 1.90	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.2
Spleen	0.7 ± 0.07	0.5 ± 0.13	2.9 ± 0.44[a,b]	0.9 ± 0.08	0.8 ± 0.2	4.6 ± 1.0[a,b]
Kidney	2.8 ± 0.08	1.8 ± 0.25	1.5 ± 0.20[a]	1.3 ± 0.07	1.0 ± 0.1	0.7 ± 0.1[a]
Diaphragm	0.7 ± 0.13	0.7 ± 0.23	0.7 ± 0.12	0.9 ± 0.2	1.2 ± 0.41	1.0 ± 0.2
Pericardium	11.0 ± 1.84	8.7 ± 1.30	15.7 ± 2.59	124.8 ± 4.2	122.0 ± 8.5	121.2 ± 3.8
Urine	18.7 ± 0.66	15.1 ± 0.99	14.9 ± 2.90			
Feces	1.6 ± 0.61	2.6 ± 0.55	2.7 ± 0.43			
Bowel	3.6 ± 1.0	2.3 ± 0.42	3.1 ± 1.05			

Method A was used for this study. The same volume (V2) of each dose (D1–D3) of avidin and ^{99m}Tc-blue biotin-liposomes was injected. Values are the mean ± SEM. Method A: avidin followed 2 h later by ^{99m}Tc-biotin-liposomes; V2: 0.06 mL avidin, 0.1 mL ^{99m}Tc-biotin-liposomes; D1: 0.5 mg avidin, 0.5 mg phospholipid; D2: 1.0 mg avidin, 1.0 mg phospholipid; D3: 5.0 mg avidin, 5.0 mg phospholipid.

$p < 0.05$, one-way ANOVA. [a]vs. D1; [b]vs. D2.

be approximately 15 μg when D1 was injected, 30 μg for D2, and 150 μg for D3. Using the values of % ID/organ presented in Table 3, it was possible to estimate that the amount of blue dye delivered to mediastinal nodes by the liposomes was four times greater when D3 was used as compared with D1 and D2 (Table 4). However, the amount of blue dye delivered to other major organs was also higher with D3 than with D1 and D2. This can clearly be seen in graphic form in Figure 5. This graph demonstrates that for estimated blue dye delivery, D1 has better mediastinal node to blood and spleen ratios as compared with D2, and a much better ratio as compared with D3. In this context, specific targeting to mediastinal nodes was much better with D1, in which the quantity of blue dye delivered to nodes was only slightly less than with D2 (Table 4). This specific delivery can be visually appreciated in Figure 6, where mediastinal nodes of rats injected with dose D1 have the most prominent ^{99m}Tc activity, whereas mediastinal node activity was less prominent in the other rats injected with D2 and D3.

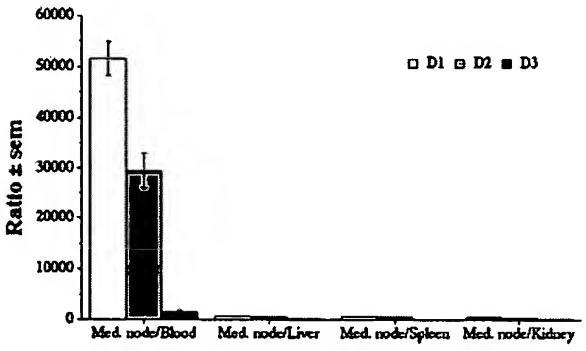

Figure 4. Ratio values of the % ID/g of ^{99m}Tc-blue-biotin-liposomes between mediastinal nodes and other organs for the injected doses, D1–D3 (D1: 0.5 mg of phospholipid and 0.5 mg of avidin; D2: 1.0 mg of phospholipid and 1.0 mg of avidin; D3: 5.0 mg of phospholipid and 5.0 mg of avidin). Volume V2 (0.1 mL of ^{99m}Tc-biotin-liposomes, 0.06 mL of avidin) was used in these injections (see text for details).

DISCUSSION

The feasibility to direct therapeutic agents to regional lymph nodes could have significant therapeutic potential. Recent efforts for drug delivery to the lymphatics have been focused on the use of colloidal particles as drug carriers.[8] Emulsions, liposomes, and nanospheres have been described as potential drug carrier systems that can provide a considerable reduction of undesirable side reactions of the free drug.[32] Subcutaneous and intraperitoneal injection of liposomal drug formulations have shown promising results for drug delivery in lymph nodes and therapeutic procedures in experimental animals.[17,18,33–35] Several

Table 4. Total Amount (in Micrograms) of Blue Dye Delivered by Liposomes in Mediastinal Nodes and Other Important Organs

	Dose (μg)/organ		
Organ	D1 ($n=4$)	D2 ($n=7$)	D3 ($n=3$)
Mediastinal nodes	2.4 ± 0.21	2.6 ± 0.36	10.4 ± 0.63
Blood	0.05 ± 0.003	0.1 ± 0.01	4.5 ± 1.8
Liver	1.3 ± 0.19	1.8 ± 0.41	12.3 ± 2.8
Spleen	0.1 ± 0.01	0.2 ± 0.04	4.4 ± 0.66
Kidney	0.4 ± 0.01	0.5 ± 0.07	2.3 ± 0.30
Pericardium	1.7 ± 0.23	2.6 ± 0.39	23.6 ± 3.9

Values are the mean ± SEM, based on the % ID/organ values presented in Table 3.
Total dose injected: D1, 15 μg; D2, 30 μg; D3, 150 μg.

liposome-based products have been approved for clinical commercial use.[36,37]

In the present work, we show the potential of another liposomal formulation, the avidin/biotin-liposome system, as a carrier system for drug delivery to mediastinal nodes using intrapleural injection as the pathway of delivery. We already have reported the potential of this system for regional lymph node targeting using subcutaneous and intraperitoneal injection.[17,18] Mediastinal nodes are involved as centers of incubation and dissemination in several diseases including lung cancer, tuberculosis, and anthrax.[19,21,22] Treatment and control of these diseases are hard to accomplish because of the limited access of drugs to mediastinal nodes using common pathways of drug delivery. Also, the anatomical location of mediastinal nodes represents a difficult target for external beam irradiation. Drug delivery using the avidin/biotin-liposome system injected intrapleurally could solve some of these limitations and offers several advantages for the treatment of these diseases. Not many attempts have been tried using the pleural space as a pathway for drug delivery to mediastinal nodes. Intrapleural injection of a liposome-entrapped chemotherapeutic platinum compound in patients with malignant pleural effusions secondary to lung cancer, malignant pleural mesothelioma, and ovarian cancer has been previously described.[38] In that work, the authors reported several therapeutic advantages of the intrapleural technique over intraperitoneal and intravenous injections of the same liposomal

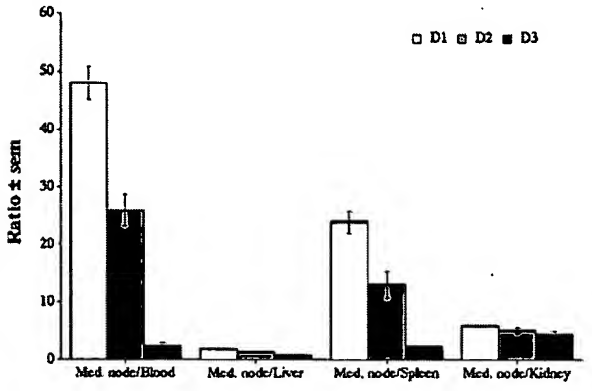

Figure 5. Ratio values of the estimated blue dye delivery between mediastinal nodes and other organs for the injected dose, D1–D3 (D1: 0.5 mg of phospholipid and 0.5 mg of avidin; D2: 1.0 mg of phospholipid and 1.0 mg of avidin; D3: 5.0 mg of phospholipid and 5.0 mg of avidin). Volume V2 (0.1 mL of ^{99m}Tc-biotin-liposomes, 0.06 mL of avidin) was used in these injections.

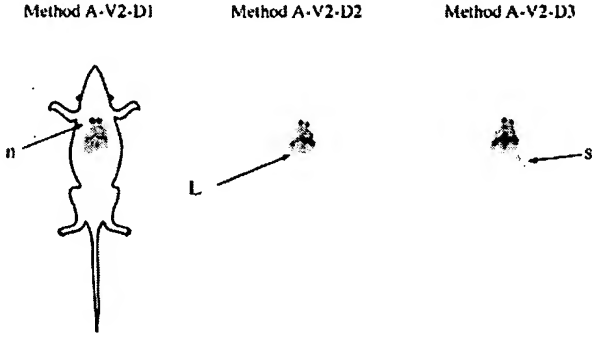

Figure 6. Scintigraphic images depicting the 22-h activity distribution of ^{99m}Tc-blue-biotin-liposomes using Method A injection procedure of the avidin/biotin-liposome system in the pleural space. An image of the three different injected doses, D1–D3 (D1: 0.5 mg of phospholipid and 0.5 mg of avidin; D2: 1.0 mg of phospholipid and 1.0 mg of avidin; D3: 5.0 mg of phospholipid and 5.0 mg of avidin) is presented. Volume V2 (0.1 mL of ^{99m}Tc-biotin-liposomes, 0.06 mL of avidin) was used in these injections. L, liver; s, spleen; and n, mediastinal nodes.

formulation and the free drug. However, no mention was made about mediastinal node targeting.

In therapeutic terms, an effective treatment of a disease affecting lymph nodes relies on an effective delivery of high concentrations of a drug to the lymph nodes. The results presented in this work (Tables 1 and 2) showed that by 22 h after injection, good retention (15.7% ID/organ; 515 % ID/g) of liposomes was achieved with the avidin/biotin-liposome system when Method A-V2 was used. This value was almost 7-fold higher than the targeting achieved when the system was intraperitoneally injected.[18] In the absence of avidin, liposomes were minimally retained in the nodes (<1.0 % ID/organ; 36% ID/g). This approach is the reverse of the sequence used in prior subcutaneous and intraperitoneal studies in which avidin was injected after the biotin-liposomes.[17,18] In terms of the injection sequence and independent of the volume injected, Method A results in the best targeting (Tables 1 and 2) of mediastinal nodes. These results confirmed our original hypothesis that an aggregate of liposomes is produced and retained in the nodes when avidin is injected some time before biotin-liposomes.

Colloid size is an important determinant for lymphatic uptake and lymph node retention when subcutaneously injected.[6,7,39] When intraperitoneally injected, colloid size does not become important within the nanometer size range and the only barrier for lymphatic uptake is the size limit of the junctions of the lymphatic wall, the mesothelial stomata.[6] Because few studies have been performed using the pleural space for lymphatic targeting, it is not totally clear what role colloid size has in lymphatic uptake with this delivery route. In pleural space, mesothelial stomata, with diameters ranging from 2 to 12 μm, are also the entrance points for lymphatic uptake.[30,40] In the present work, it was found that when a large aggregate of liposomes is formed in the pleural space it is not easily removed by the lymphatics, and only when the aggregate is formed in the lymphatics, can retention in lymph nodes be observed. Hirano and Hunt[12] have reported that medium-size liposomes (250–400 nm) show better retention in mediastinal nodes after intraperitoneal injection, but they also concluded that liposome stability *in vitro* and *in vivo* decreases with increasing liposome size. An advantage of the avidin/biotin-liposome system injected as described here is that it is possible to use small liposomes (100–200 nm), which are very stable for drug targeting to mediastinal nodes. In this way, liposome size would not be an important factor for mediastinal node targeting; however, for comparison, a more detailed study of lymphatic targeting using intrapleural injection of different size liposomes should be pursued.

Liposome retention in lymph nodes is explained by two different ways: by a simple mechanical filtration, or through phagocytosis by the reticuloendothelial system cells.[6] When the avidin/biotin-liposome system is used, it is possible that a combination of both ways of retention take place. When avidin is first injected, it moves quickly into the lymphatic vessels that drain the pleural space and some avidin is retained into the nodes, probably due to either simple pooling in the lymph node vasculature or by retention of the positively charged avidin[41] on the negatively charged surface of the lymphatic vessels within the lymph node.[25] Then when the biotin-liposomes are injected 2 h later, they move into the lymphatics and an aggregate is created. If some biotin-liposomes move into the nodes without interacting with the avidin, once in the nodes they can potentially still interact with the avidin that is already retained there and produce some aggregates within the nodes. Once the aggregate is collected in the nodes, simple mechanical filtration of these large aggregates could be the means by which the liposomes are retained in the nodes. In this way, when injected first and before the biotin-liposomes, avidin prevents the biotin-liposomes from gaining fast access to the blood circulation.

When biotin-liposomes are injected first, they leave the pleural space and move into the lymphatics during the first 2 h without showing any significant retention into the nodes (Fig. 3). Then, when avidin is injected and begins to move into the lymphatics, an aggregate is produced with the biotin-liposomes that have already moved into the lymphatics. Because not all the volume of the injected agents is removed from the pleural space within 2 h,[25] it appears that some aggregate is still produced in the pleural space with both injection sequences. These aggregates could result in a prolonged targeted sustained depot-like delivery of liposomes to the lymphatics.

In terms of the injected volume, it has been reported that an increased volume of fluid injected into the pleural space increases the rate of lymphatic drainage, which favors fluid removal from pleural space.[42] However, in terms of mediastinal node targeting, the results obtained in the present work indicated that the volume injected in pleural space does not affect the nodal targeting, despite

that an interaction-effect between method and volume was observed with the two-way ANOVA. The more significant effect in terms of the injection of a large volume in the pleura was the presence of some liposome aggregates in the pleural space observed during the 22-h biodistribution studies. This observation was more evident when compared with the other groups in which less volume was injected. Apparently, it is possible that the injected volume was so large that it was not totally removed from the pleural space after 2 h; in consequence, when the second agent was injected, some large liposome aggregates were produced in the pleural space. It is also possible that the injection of large volumes of the agents could produce so many liposome aggregates that they block the lymphatic channels that drain into the mediastinal nodes, resulting in a decreased uptake into the nodes.

In terms of the dose injected, the results showed that a high dose of liposomes injected in the pleural space does not result in a high accumulation in mediastinal nodes (Table 3). Injection of a small dose results in a more specific mediastinal node targeting as demonstrated by the ratios of the % ID/g in mediastinal nodes and other organs. Despite the fact that "drug" (blue dye) delivery in mediastinal nodes was higher when a large dose was injected (Table 4), the delivery to other organs was also high as compared with small-dose injection (Fig. 5). These results may have important therapeutic significance in terms of decreasing drug delivery to healthy tissue in order to avoid side effects.

The present study was performed using normal animals. Thus, pharmacokinetics, targeting, and drug delivery using intrapleural injection of the avidin/biotin-liposome system could change under abnormal pathological conditions because lymphatic channels and nodes could be obstructed in an advanced disease process.[5] Future studies should be performed using various disease models with nodal involvement to compare the results observed in this work.

The specific targeting of a liposome-encapsulated drug to mediastinal lymph nodes could result in a prolonged targeted sustained depot-like delivery of high drug concentrations to these nodes while the liposomes are slowly degraded and metabolized by phagocytic cells located within these nodes. Future experiments using intrapleural injection of the avidin/biotin-liposome system to target drugs to mediastinal nodes should be pursued.

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Mediastinal Node and Diaphragmatic Targeting after Intracavitary Injection of Avidin/^{99m}Tc-Blue-Biotin-Liposome System

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ABSTRACT: A method for delivering drugs to sites of disease extension in mediastinal nodes is described. Mediastinal node and lymphatic distributions were determined after intracavitary injection of the avidin/biotin-liposome system in normal rats. The effect of the injected dose on lymphatic targeting of liposomes after intraperitoneal injection of ^{99m}Tc-blue-biotin-liposomes and intrapleural injection of avidin, and vice versa, is presented. Scintigraphic imaging was used to follow the movement of ^{99m}Tc-blue-biotin-liposomes to determine the pharmacokinetics and organ uptake. Tissue biodistribution studies were performed 22 h after injection of the ^{99m}Tc-blue-biotin-liposomes. Results indicated that independent of the cavity in which each agent was injected, a dose of 5.0 mg of each agent results in higher mediastinal node targeting (8%–10% ID/Organ) as compared with the injection of a 0.5 mg dose (2%–5% ID/Organ, $p < 0.05$). Targeting of diaphragm and associated lymphatics was observed when ^{99m}Tc-blue-biotin-liposomes were injected in peritoneum and avidin in pleural space. In contrast, pleural, and pericardial lymphatic targeting was observed when ^{99m}Tc-blue-biotin-liposomes were injected in pleural space and avidin in peritoneum. Intracavitary injection of the avidin/biotin-liposome system could potentially be used for the delivery of prophylactic drugs that could reduce tumor metastasis and infection spread to mediastinal nodes. J Pharm Sci 94:1–18, 2005

Keywords: liposomes; drug targeting; pharmacokinetics; lymphatic transport; scintigraphy; nanoparticles; technetium-99m; avidin-biotin system; pleural space; peritoneum

INTRODUCTION

Drug delivery to the lymph nodes has been described as a procedure with great potential for the treatment and control of disease processes that involve lymphatic spread into other locations of the body.[1,2] Infection and tumor spread from peritoneal cavity into the thoracic and pleural cavities via the lymphatics has been previously described.[3–5] This spread is possible because of the specialized lymphatic channels that drain fluid from the peritoneal cavity.[6] Peritoneal fluid enters lymphatic lacunae located beneath the peritoneum of the diaphragm via openings between mesothelial cells (stomata) covering the surface of the diaphragm. From there, another group of collecting lymphatics located beneath the pleura covering the diaphragm drain fluid into parasternal lymphatics. These lymphatic vessels carry the fluid into the superior mediastinal nodes, which filter the fluid before entering the vascular system via the right lymphatic or the thoracic duct.[6,7]

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Under normal conditions, the peritoneum deals with a disease process in three ways: (i) direct absorption of the disease agent into the lymphatics and lymph nodes that filter and destroy the agent before it can access the vascular system; (ii) local destruction of the disease agent through phagocytosis by either local macrophages or polymorphonuclear granulocytes; and (iii) localization of an infection in the form of an abscess.[3] However, for an advanced disease process, the peritoneum is not always able to control the disease and the disease spreads into other regions via the lymphatics as in the case of systemic sepsis or ovarian and gastric cancer, and peritoneal carcinomatosis.

For the treatment of peritoneal disease processes, liposomal formulations of several therapeutic drugs have been used in previous clinical and experimental trials with promising therapeutic results.[8–12] Liposomes (lipid nanoparticles) as drug carriers, have numerous advantages over other carrier systems including: (1) they can transport water-soluble or lipid-soluble drugs and high-molecular weight substances and release them in a sustained manner; (2) because the lipids used to form the liposomes are natural components of the organism, antigenicity, and toxicity of liposomes are very low; (3) biodistribution of liposomes can be controlled in terms of the size and lipid composition; (4) antibodies and chemical compounds can be attached to the surface of liposomes to improve their affinity for specific targets; and (5) to reduce clearance of liposomes from the bloodstream and their affinity for the reticuloendothelial system (RES), liposomes can be modified with polyethylene glycol (PEG).[12–15]

In the present work, we evaluated the potential of a previously developed avidin/biotin-liposome system for targeting mediastinal nodes and the lymphatics that drain the peritoneum and pleural space. This was performed by injecting one component, either avidin or biotin-liposomes, into the peritoneal cavity and the corresponding component in the pleural space, and vice versa. Our interest was to determine whether this system can be used as a delivery system for drugs and therapeutic radionuclides as a method to control and limit disease spread by producing a significantly greater retention of liposomes in lymphatics channels and mediastinal nodes while avoiding liposome aggregation in the cavity of injection. In a previous work, the potential of this system for drug delivery in the peritoneum and associated lymph nodes by injecting both components in the peritoneal cavity was reported.[16] More recently, we have shown the potential of this system for targeting mediastinal nodes after injection of both components in the pleural space.[17] In the present work, we determined that when one component is injected in peritoneum and the other component is injected in the pleural space, significant differences could be observed in terms of liposome retention in diaphragm, the collecting lymphatics that cover the diaphragm, and the pleural and thoracic lymphatics. However, no significant differences were observed in terms of mediastinal node targeting. It was observed that the injected dose of the agents had a significant effect on lymphatic targeting and organ biodistribution of the liposomes delivered by these intracavitary injection routes. These results have important significance in terms of the potential delivery of prophylactic drugs in the lymphatics that could reduce or eliminate disseminating infectious agents or tumor cells in the process of invading other regions of the body via these lymphatics.

To perform this study, scintigraphic images were acquired with a gamma camera to noninvasively follow the pharmacokinetics and organuptake of the avidin/biotin-liposome system. Liposomes, containing blue dye in their interior space to emulate the process of drug delivery, were labeled with technetium-99m (^{99m}Tc). The encapsulation of blue dye permitted visualization of the lymph nodes and lymphatic channels during the biodistribution studies performed at the end of each study at 22 h after injection of the ^{99m}Tc-blue-biotin-liposomes.

MATERIALS AND METHODS

Preparation and Characterization of Blue-Biotin-Liposomes

To follow the pharmacokinetics and organ uptake of the avidin/biotin-liposome system with a scintigraphic camera, the biotin-liposomes were labeled with ^{99m}Tc.[18] Before ^{99m}Tc labeling, reduced glutathione (GSH) in PBS, pH 6.3, and patent blue dye were encapsulated in the liposomes. The lipid composition of liposomes includes a phospholipid with biotin conjugated to the head group region.[19]

Preparation of Blue-Biotin-Liposomes

Biotin-coated liposomes were comprised of distearoyl phosphatidylcholine (Avanti Polar Lipids, Pelham, AL)/cholesterol (Calbiochem, San Diego, CA)/*N*-biotinoyl distearoyl phosphoethanolamine

(Northern Lipids, Vancouver, Canada)/α-tocopherol (Aldrich, Milwaukee, WI) (58:39:1:2 total lipid molar percentage). Lipid ingredients were codried from chloroform (Fisher Scientific, Fair Lawn, NJ) by removing it by rotary evaporation and desiccation for 24 h. The dried lipid film was rehydrated with 300 mM sucrose (Sigma, St. Louis, MO) in sterile water (at a total lipid concentration of 120 μmol/mL), warmed to 55°C, and then lyophilized overnight. The dried lipid-sucrose mixture was rehydrated with 200 mM GSH (Sigma) in Dulbecco's phosphate buffered saline (PBS) (pH 6.3) and 10 mg/mL patent blue dye (CI 42045; Sigma). Before extrusion, the lipid suspension was diluted to 40 μmol/mL with 100 mM GSH in PBS containing 150 mM sucrose and 10 mg/mL patent blue dye and then extruded through a series of polycarbonate filters (Lipex Extruder, Vancouver, Canada) (2 μm, two passes; 400 nm, two passes; 100 nm, five passes) at 55°C to form unilamellar liposomes. Unencapsulated GSH, blue dye, and sucrose were then removed by repeated washings and ultracentrifugation at 41000 rpm for 50 min (Ti 50.2 rotor; Beckman, Fullerton, CA). Liposomal pellets were resuspended in PBS containing 300 mM sucrose (pH 6.3) to a total lipid concentration of 60 μmol/mL and stored at 4°C until needed.

Characterization of Liposomes

Liposomes were tested for liposomal size by determining the diameter and polydispersity index (PI) using particle size analyzer (Brookhaven Instruments, Holtsville, NY). The PI measures the homogeneity of the liposome sample with regard to diameter. A value of 0.0 determines a completely homogeneous sample while a value of 1.0 determines a completely heterogeneous sample. Liposomes were also tested for phospholipid content by Stewart assay;[20] biotin-phospholipid content by 2-(4′-hydroxyazobenzene) benzoic acid (HABA) assay (Pierce, Rockford, IL); GSH content by BIOXYTECH GSH-400 assay (R&D Systems, Minneapolis, MN); blue dye concentration by spectrophotometry; and sterility and endotoxin levels. Results represent the average of three-to-four trials for each test. GSH, blue dye, and biotin-phospholipid were released from liposomes by detergent solubilization before performing assays.

Liposome Solubilization

To solubilize liposomes, 100 μL of the blue-biotin-liposomes was mixed with 100 μL of 10% (w/v) *n*-octyl-β-D-glucopyranoside (OBG) (Sigma) in PBS, pH 6.3, and incubated for 30 min at 37°C.

Biotin Determination

(i) Prior to determination of the biotin content the HABA-avidin reagent was prepared. HABA (10 mM in 10 mM NaOH) was prepared by adding 24.4 mg of HABA to 9.9 mL of PBS (pH 6.3) and 0.1 mL NaOH (1 M). Then, the HABA solution was mixed with 10 mg avidin (Sigma) to form the HABA-avidin reagent. This reagent was stored at 4°C for up to 2 weeks until used.

(ii) A 200 μL aliquot of OBG solubilized liposomes were mixed with 900 μL ice-cold solution of HABA-avidin reagent and centrifuged at 13000 rpm for 10 min at room temperature. After centrifugation, the supernatant was separated from the pellet and stored at 4°C until used and the pellet was discarded.

(iii) A 1.0 mL aliquot of the supernatant was used to determine the amount of biotin by monitoring absorbance differences of HABA-avidin reagent in the presence and absence of biotin-phospholipid using spectrophotometry at 500 nm. When biotin is present, the absorption is decreased in proportion to the amount of biotin[21] because the biotin displaces the HABA dye due to its higher affinity for avidin. The amount of biotin present in the sample was calculated from a modified method using the formula proposed by Green:[21]

$$\text{Biotin (mM)} = \frac{(A_1 - A_2 C_f)}{34};$$

where A_1 is the absorbance of the standard HABA-avidin reagent; A_2 is the absorbance of the supernatant containing avidin-biotin-phospholipid; and C_f is a correction factor for HABA dilution and changes in absorbance because of the presence of OBG. Previous pilot experiments revealed that OBG does not affect the absorbance signal and since the dilution of HABA was minimal, $C_f = 1.0$ was used. It was found that the presence of blue dye did not interfere with the measurement of biotin because the blue dye absorbance peak is observed at 637 nm.[22]

GSH and Blue Dye Determination

The same 1 mL-aliquot of supernatant used to measure the biotin content also was used to determine GSH and blue dye concentration. GSH was separated from blue dye, HABA, and OBG by using a D-salt Polyacrylamide 1800 Desalting Gel column (Pierce). One milliliter of the supernatant was loaded on the D-salt column and eluted with PBS (pH 6.3). Twenty-two 0.5 mL fractions were collected from the column in plastic cuvettes. All fractions were checked by visual inspection, spectrophotometry at 500 and 637 nm, and assayed using BIOXYTECH GSH-400 kit. By measuring GSH content in fractions 1–10 individually, we determined that GSH elutes primarily in fractions 5–8. Fractions 5–8 were used to calculate the total amount of GSH content in liposomes. Blue dye concentration was calculated spectrophotometrically at 637 nm using fractions 11–22, which could easily be visualized due to blue color. From a calibration curve of standard blue dye solution (0.0–5.0 mg/mL), the amount of dye in each fraction was calculated and the total amount of dye was determined by summing these values. As mentioned above, we found that the presence of HABA and OBG did not affect the measurement of blue dye concentration.

Liposomes were 120 nm (0.024 PI) in diameter and contained 37.5 mmol/L of phospholipid and 2.1 mmol/L GSH. Concentration of blue dye was 0.84 mg/mL. No growth of bacteria was detected and the endotoxin level was >25 and <250 EU/mL.

Liposome Labeling Procedure

Labeling of blue-biotin-liposomes with ^{99m}Tc was performed as described before.[18] Briefly, a commercial kit of lipophilic chelator, hexamethylpropyleneamine oxime (HMPAO, Ceretec, GE Healthcare, Arlington Heights, IL), was reconstituted with either 5 mL of saline containing 555 MBq (15 mCi) or 2.5 mL containing 2775 MBq (75 mCi) of ^{99m}Tc-pertechnetate (GE Healthcare Radiopharmacy, San Antonio, TX) depending on the final specific activity of the liposomes needed when small volume injections were used. An aliquot of ^{99m}Tc-HMPAO was added to a concentrated suspension of blue-biotin-liposomes (1:1 ratio) encapsulating GSH and incubated at room temperature for 30 min. Free label was removed from the liposomes by passage over Sephadex G-25 column (Amersham Pharmacia Biotech, Uppsala, Sweden). Labeling efficiencies were checked by determining the ^{99m}Tc activity before and after column separation of ^{99m}Tc-blue-biotin-liposomes using a dose calibrator (Mark 5: Radix, Houston, TX). The labeling efficiency in all the experiments was between 75% and 85%.

Animal Studies

Normal male Sprague–Dawley rats (0.25–0.35 kg) were used in these experiments. All the experiments were performed under the National Institutes of Health Animal Use and Care Guidelines and approved by the University of Texas Health Science Center at San Antonio animal use committee. Before performing any imaging procedures and euthanasia, the animals were anesthetized by inhalation with Isoflurane-USP (VEDCO, Inc., St. Joseph, MO) using an anesthesia inhalation machine (Backford, Inc., Wales Center, NY) (2.5 L/min oxygen at 147 PSIA; 3% Isoflurane).

Intraperitoneal Injections (IP)

The abdominal region of the rat was shaved and the injection was performed in the lower left quadrant of the abdomen using a 20-G 1.25 inch angiocatheter (Becton Dickinson, Sandy, UT). To confirm penetration, 0.5 mL of saline was injected through angiocatheter and aspirated to confirm that neither the urinary bladder, intestine nor blood vessel had been entered. If the catheter was correctly placed, then the syringe with either avidin or ^{99m}Tc-blue-biotin-liposomes was fitted to the catheter and the volume was injected.

Intrapleural Injections (Pleu)

Injections in the pleural space were performed using the following technique. Under anesthesia, rats were shaved in the lateral left chest. An incision of approximately 8 mm was made through the skin, then the fascia was dissected away and a small incision was made through the external oblique muscle layer, the latissimus dorsi and the serratus layers. Using fine scissors, a nick was made in the intercostal layers. The intercostal layers were punctured using a flat tipped (19 G) needle stub (~4.5 mm in length). To confirm penetration and to prevent damage to the underlying lungs, a 1 mL tuberculin syringe was fitted to the 19-G luer hub and 0.1 mL of air was injected into the pleural space. When successfully placed, the air will enter the pleural space without resistance. If resistance was encountered, the 19-G stub was removed and reintroduced again.

The material was then injected using a flat tipped 23-G needle stub (~20 mm in length) inserted through the 19 G stub.

Study Design

Eight groups of rats, four experimental and four control (3–5/gp), were used in two methods of injection to evaluate mediastinal node targeting and organ biodistribution (Fig. 1). In each method two different doses (0.5 and 5.0 mg) were studied and the mg dose of avidin and phospholipid were equal in each group studied.

1. IP/Pleu Method: ^{99m}Tc-blue-biotin-liposomes were injected IP and avidin was injected intrapleurally. Experimental groups are denoted IP/Pleu(0.5) and IP/Pleu(5.0), and corresponding control groups are denoted IP/Pleu(0.5)c and IP/Pleu(5.0)c.
2. Pleu-IP Method: ^{99m}Tc-blue-biotin-liposomes were injected intrapleurally and avidin was injected IP. Experimental groups are denoted as Pleu/IP(0.5) and Pleu/IP(5.0), and corresponding control groups are denoted as Pleu/IP(0.5)c and Pleu/IP(5.0)c.

To obtain the 0.5 mg dose of liposomes, an aliquot of the original stock of liposomes was diluted in PBS (pH 6.3) and adjusted to get the required dose. The 5.0 mg dose was obtained by concentrating an aliquot of the stock liposomes to the required dose by ultracentrifugation. These doses and the volume injected for either IP or Pleu method were empirically determined from previous experiments.[16,17] It was observed that for IP injections, the volume injected could affect the rate of fluid removal from peritoneum, but for intrapleural injections, the volume injected does not significantly affect the rate of lymphatic absorption and mediastinal node targeting from pleural space. For this reason, a constant volume of 1.0 mL, plus the 0.5 mL used to verify catheter location, was used during IP injections. From previous studies, we determined that the volume of 0.3 or 0.03 mL injected in pleural space of normal rats results in the same rate of fluid removal.[17]

	Biotin-liposomes	5 min	Avidin		Imaging at		Biodistributions
IP/Pleu(0.5)	0.5 mg/1.0 ml	→	0.5 mg/0.03 ml	→	baseline,	→	at
	injected IP		injected in pleura		2, 4, 6, 12, and 22 h		22 h
	Biotin-liposomes	5 min	Avidin		Imaging at		Biodistributions
IP/Pleu(5.0)	5.0 mg/1.0 ml	→	5.0 mg/0.3 ml	→	baseline,	→	at
	injected IP		injected in pleura		2, 4, 6, 12, and 22 h		22 h
	Biotin-liposomes	5 min	Avidin		Imaging at		Biodistributions
Pleu/IP(0.5)	0.5 mg/0.03 ml	→	0.5 mg/1.0 ml	→	baseline,	→	at
	injected in pleura		injected IP		2, 4, 6, 12, and 22 h		22 h
	Biotin-liposomes	5 min	Avidin		Imaging at		Biodistributions
Pleu/IP(5.0)	5.0 mg/0.3 ml	→	5.0 mg/1.0 ml	→	baseline,	→	at
	injected in pleura		injected IP		2, 4, 6, 12, and 22 h		22 h
	For control groups saline was injected instead of avidin (using same volumes).						
	From previous studies we determined that a volume of 0.3 or 0.03 ml injected in pleural space of normal rats results in the same rate of fluid removal.						

Figure 1. Experimental design for the intracavitary injection of the avidin/biotin-liposome system where one component was injected intraperitoneally (IP) and the other component was injected intrapleurally (Pleu) and vice versa. The components were tested at two different doses (0.5 or 5.0 mg). For clarification, the first route specified in the IP/Pleu or Pleu/IP nomenclature is the ^{99m}Tc-blue-biotin-liposomes and the second is the avidin.

IP-Pleu Method

Two experimental groups (IP/Pleu(0.5), IP/Pleu(5.0)) were used to perform this study. For the first group, a 0.5 mg dose of ^{99m}Tc-blue-biotin-liposomes (1.67 mg of phospholipid/kg-body wt, 13 MBq) diluted in 1.0 mL saline was injected in the peritoneum and the same dose of avidin (0.5 mg) diluted in 0.03 mL saline was injected in the pleural space. For the second group, a 5.0 mg dose of the concentrated ^{99m}Tc-blue-biotin-liposomes (16.7 mg of phospholipid/kg-body wt, 12 MBq) diluted in 1.0 mL saline was injected IP, and the same dose of avidin diluted in 0.3 mL was injected in pleural space. A control group denoted as IP/Pleu(0.5)c and IP/Pleu(5.0)c was performed for each group using the same doses of ^{99m}Tc-blue-biotin-liposomes, but saline (0.03 or 0.3 mL) was injected instead of avidin.

Pleu-IP Method

For first group Pleu/IP(0.5), a 0.5 mg dose of ^{99m}Tc-blue-biotin-liposomes (1.67 mg of phospholipid/kg-body wt, 7 MBq) diluted in 0.03 mL saline was injected in pleural space and the same dose (0.5 mg) of avidin diluted in 1.0 mL saline was injected IP. For the second dose group, Pleu/IP(5.0), a 5.0 mg dose of ^{99m}Tc-blue-biotin-liposomes (16.7 mg of phospholipid/kg-body wt, 29 MBq) diluted in 0.3 mL saline was injected in pleural space and 5.0 mg avidin diluted in 1.0 mL saline was injected in peritoneum. For control groups (Pleu/IP(0.5)c and Pleu/IP(5.0)c), experiments were performed in a similar manner except saline was injected instead of avidin.

Image Acquisition

Static scintigraphic images were acquired in a 128×128 Word Image Matrix with a zoom of $1.45\times$ using a Picker SX-300 gamma camera (Cleveland, OH) interfaced to a Medasys Pinnacle computer (Miami, FL). Images for the ^{99m}Tc-blue-biotin-liposomes were acquired in the ^{99m}Tc window (140 keV $\times$ 20%) using a Low Energy High Resolution collimator.

Biodistributions

Biodistribution studies were performed 22 h after ^{99m}Tc-blue-biotin-liposome injection. After imaging, the anesthetized animals were euthanized by cervical dislocation, and tissues were harvested, weighed, and counted for radioactivity (Auto-Gamma 5000 Series, Packard Instruments, Downers Grove, IL). The percentage of injected dose (%ID) per organ and per gram of tissue was calculated by comparison with a standard aliquot of the radioactive material used.

Image Analysis

Regions of interest (ROI) were drawn around the mediastinal nodes and selected organs on the images using Osiris Imaging Software (Version 3.1, University Hospital of Geneva, Geneva, Switzerland). A box was drawn around the entire body in the baseline image to determine the number of counts injected into the body (A_0). The % of activity at some specific time t (%$A(t)$) accumulated in the ROI was calculated as the ratio of the counts in the ROI ($A_{ROI}(t)$) and the total number of counts detected in the whole body at baseline (A_0), multiplied by 100 ($\%\ A(t) = A_{ROI}(t)/A_0 \times 100$). Counts associated with the ROI's were corrected for radioactive decay (referred to the injection time), the image acquisition time, and for background activity when the image acquisition time was longer than 15 min.

Statistical Analysis

Values are reported as mean $\pm$ SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) to compare the % of injected dose per organ (%ID/organ) and %ID/g in the mediastinal nodes and other organs using SPSS Base 10.0 software (SPSS, Inc., Chicago, IL). Rats were considered nested between method-dose groups. Differences in %ID/organ and %ID/g were statistically compared using between-groups multiple comparisons. When necessary, comparisons among means were Bonferroni adjusted. A log transformation was applied to the data to better satisfy the assumptions underlying the analysis. Means and standard errors were computed from untransformed data and statements of statistical significance were based on transformed data. $p < 0.05$ was defined as an acceptable probability for a significant difference between means.

RESULTS

Image Analysis and Pharmacokinetics

Figure 2 shows the 22 h scintigraphic images for experimental and control groups for each method of study: ^{99m}Tc-blue-biotin-liposomes injected IP and avidin injected in pleural space (IP/Pleu) and

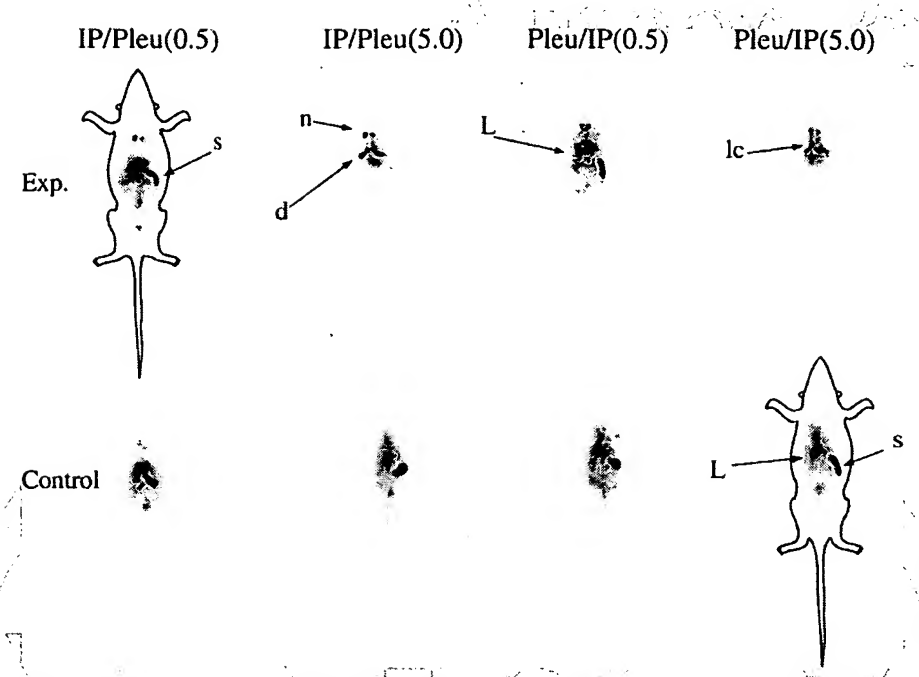

Figure 2. Scintigraphic images of rats at 22 h depicting mediastinal node accumulation and organ distribution after injection in either peritoneal (IP) or pleural space (Pleu) of ^{99m}Tc-blue-biotin-liposomes alone (Control) or following injection of avidin in the corresponding cavity (Exp). Images depict accumulation of the ^{99m}Tc-blue-biotin-liposomes in the mediastinal nodes (n), diaphragm (d), liver (L), lymphatic channels (lc), and spleen (s).

vice versa (Pleu/IP). The results show that when no avidin is injected, liposomes leave the injection site (either pleural space or abdominal cavity), move into blood circulation and eventually collect in spleen and liver with minimal accumulation in mediastinal nodes or collecting lymphatics. However, when avidin is injected, accumulation in mediastinal nodes, diaphragm, and collecting lymphatics in the pleura that cover the diaphragm is observed.

In reference to the injected dose, it was observed that when the 0.5 mg dose was used in either IP/Pleu or Pleu/IP method, accumulation of liposomes in mediastinal nodes and pleural lymphatics was minimal as compared with the other two groups when the 5.0 mg dose was used. Also, except for the accumulation in mediastinal nodes, no significant differences between the experimental groups injected with the 0.5 mg dose and the control groups were detected in terms of liposome distribution in blood circulation, spleen, and liver. The image results clearly show a higher accumulation in mediastinal nodes, associated lymphatics, and less uptake in liver and spleen when the 5.0 mg dose is injected.

In terms of the method of injection, Figure 3 shows scintigraphic images of the same rat acquired at several time points for comparison of the pharmacokinetics of the ^{99m}Tc-blue-biotin-liposomes when the avidin/biotin-liposome system is used with methods IP/Pleu and Pleu/IP at the 5.0 mg dose. No significant differences were observed in terms of mediastinal node targeting, and the more significant differences between both methods were related with the pharmacokinetics and the lymphatic distribution of the liposomes. When IP/Pleu method is used, liposomes move quickly from abdominal cavity into diaphragmatic lymphatics, the parasternal lymphatics in chest wall, and mediastinal nodes. When the Pleu/IP method is used, in the early images it is hard to differentiate between accumulation in mediastinal nodes and the pleural lymphatics because of the overlying activity in the thoracic cavity in the images, but in the late images it was observed that intrapleural biotin-liposomes drain into the pleural lymphatics that cover the diaphragm, thoracic lymphatics and mediastinal nodes.

For both injection methods using the 5.0 mg dose, good retention of liposomes in the

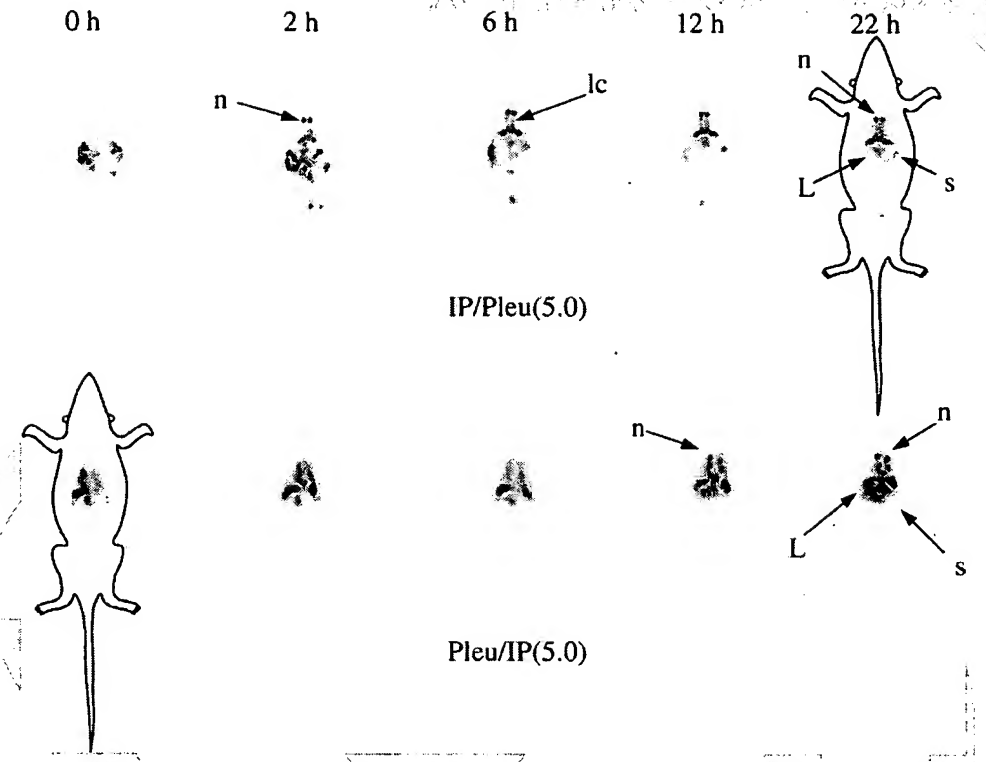

Figure 3. Scintigraphic images of rats acquired at various times after IP or intrapleural injection of ^{99m}Tc-blue-biotin-liposomes and avidin using the IP/Pleu(5.0) and Pleu/IP(5.0) methods of injection. Images depict the pharmacokinetics and organ distribution of the ^{99m}Tc-blue-biotin-liposomes in the mediastinal nodes (n), lymphatic channels (lc), liver (L) and spleen (s).

mediastinal nodes and reduced accumulation of liposomes in blood circulation, liver, and spleen after 22 h was evident. When injected IP, ^{99m}Tc-blue-biotin-liposomes moved rapidly from the abdominal cavity and into the thoracic lymphatics where they aggregated in the presence of the intrapleurally injected avidin. When injected in the pleural space biotin-liposomes were removed more slowly from the thoracic cavity because they were trapped after their interaction with the IP injected avidin that had moved quickly into the thoracic lymphatics.

Figure 4 depicts the pharmacokinetic results of the ^{99m}Tc-blue-biotin-liposomes in terms of the activity detected in mediastinal nodes, cavity of injection, spleen, and diaphragmatic lymphatics at different times by comparing method of injection and dose injected for each experimental group. Independent of the injected dose, movement of ^{99m}Tc-blue-biotin-liposomes into mediastinal nodes was faster with IP/Pleu method than Pleu/IP method (Fig. 4A). However after 12 h, the mediastinal node retention is approximately two-fold higher when 5.0 mg dose was used than when 0.5 mg dose was used independent of either IP/Pleu or Pleu/IP method.

Figure 4B describes the kinetics of the ^{99m}Tc-blue-biotin-liposomes leaving the cavity in which they were injected. The results indicate that liposomes injected in peritoneum (IP/Pleu method) are cleared rapidly from the cavity via the peritoneal lymphatics in the diaphragm. Two hours after injection, around 25% of the injected liposomes already have left the peritoneum, and after 12 h around 75% have cleared. The same behavior was observed with Pleu/IP(0.5) method. It seems likely that this small dose of avidin and biotin-liposomes injected by Pleu/IP method did not produce a significant aggregation of liposomes that could be retained in the nodes and lymphatics, and that the biotin-liposomes are just passing through the nodes and moving into blood circulation. In contrast, when the 5.0 mg dose of liposomes is injected intrapleurally, better retention of liposomes in the thoracic cavity was observed. Around 10% of the biotin-liposomes have left the

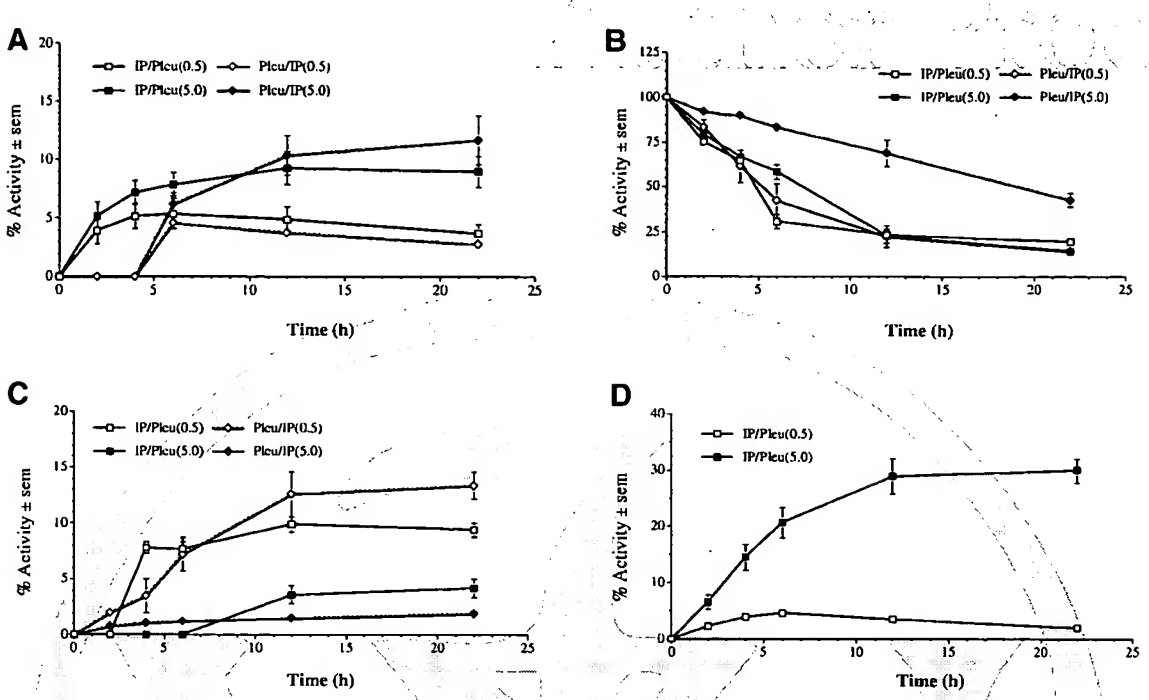

Figure 4. Image analysis results describing the pharmacokinetics of ^{99m}Tc-blue-biotin-liposomes in (A) mediastinal nodes, (B) injection cavity, (C) spleen, and (D) diaphragmatic lymphatics for experimental groups where ^{99m}Tc-blue-biotin-liposomes were injected either IP or intrapleurally (Pleu) and avidin was injected in the corresponding cavity.

thoracic cavity after 2 h, and around 25% after 12 h. It appears that when avidin is injected in peritoneum, it moves very fast into mediastinal nodes and pleural lymphatics preventing the biotin-liposomes injected intrapleurally from gaining fast access to the blood circulation.

Figure 4C describes the activity detected in spleen at several time points. The analysis indicated that the 0.5 mg dose, injected either IP/Pleu or Pleu/IP results in a fast accumulation of ^{99m}Tc-blue-biotin-liposomes by spleen, around 7% after 6 h, and around 10% after 12 h. In contrast, when the 5.0 mg dose is used, accumulation of ^{99m}Tc-blue-biotin-liposomes in spleen was reduced. It was not possible to follow the pharmacokinetics of the biotin-liposomes in liver because it was not easy to differentiate with detail its shape in the images.

Figure 4D describes the accumulation of ^{99m}Tc-blue-biotin-liposomes in the diaphragm and the collecting lymphatics located at the pleura that covers the diaphragm, when biotin-liposomes are injected IP. When the 0.5 mg dose is injected, the biotin-liposomes pass through the diaphragmatic lymphatics, <5% accumulation at any time. But when the larger 5.0 mg dose is used, more liposome aggregation is produced increasing the retention of the liposomes in those lymphatic channels, 5% after 2 h and 25% after 12 h.

Biodistribution Results

Table 1 shows the 22 h biodistribution results for experimental and control groups in terms of the % injected dose per organ (%ID/organ). In terms of the injected dose, it was observed that independent of the method of injection, the 5.0 mg dose results in high-mediastinal node targeting and better organ distribution as compared with the injection of the 0.5 mg dose. Higher accumulation of liposomes was observed in mediastinal nodes with methods IP/Pleu(5.0) and Pleu/IP(5.0), but only IP/Pleu(5.0) method was significantly different ($p < 0.05$) from methods where the 0.5 mg dose was injected. Mediastinal node accumulation of liposomes in control groups was lower than accumulation in experimental groups ($p < 0.05$), showing the potential of the avidin/biotin-liposome system in mediastinal node targeting as compared with the injection of biotin-liposomes alone. For blood, accumulation of ^{99m}Tc-blue-biotin-liposomes was significantly

Table 1. Biodistribution Results (%ID/Organ), 22 h after Injection of ^{99m}Tc-Blue-Biotin-Liposomes and Avidin in Peritoneum and Pleural Space

	IP-Pleu				Pleu-IP			
	(0.5) ($n=5$)	(0.5)c ($n=3$)	(5.0) ($n=4$)	(5.0)c ($n=3$)	(0.5) ($n=4$)	(0.5)c ($n=3$)	(5.0) ($n=4$)	(5.0)c ($n=3$)
Organ	%ID/Organ				%ID/Organ			
Blood	$9.3 \pm 1.7^{b,d}$	$12.7 \pm 4.8^{b,d}$	$4.0 \pm 2.0^{a,c}$	$16.4 \pm 2.0^{b,d}$	$10.8 \pm 2.3^{b,d}$	$19.2 \pm 3.6^{b,d}$	$0.45 \pm 0.1^{a,c}$	$18.0 \pm 0.1^{b,d}$
Heart	0.13 ± 0.01	0.23 ± 0.06	0.05 ± 0.02	0.14 ± 0.01	0.07 ± 0.03	0.3 ± 0.01	0.06 ± 0.02	0.2 ± 0.02
Lung	0.35 ± 0.04	0.56 ± 0.1	0.22 ± 0.04	0.62 ± 0.17	0.70 ± 0.09	1.5 ± 0.1	0.44 ± 0.07	0.66 ± 0.08
Thymus	0.18 ± 0.06	0.17 ± 0.02	n/c	n/c	0.6 ± 0.1	1.8 ± 0.5	0.21 ± 0.04	0.39 ± 0.07
Mediastinal nodes	$5.0 \pm 0.4^{b,c}$	$0.6 \pm 0.1^{a,b,c,d}$	$10.0 \pm 1.0^{a,c}$	$0.2 \pm 0.04^{a,b,c,d}$	$2.0 \pm 0.3^{a,b,d}$	$1.3 \pm 0.3^{a,b,d}$	8.0 ± 0.5^{c}	$0.8 \pm 0.1^{a,b,c,d}$
Liver	20.1 ± 2.2	17.7 ± 1.5	14.7 ± 3.5	10.5 ± 1.6	20.8 ± 1.7	18.9 ± 1.6	22.9 ± 4.7	9.4 ± 1.5
Spleen	$13.1 \pm 1.9^{b,d}$	$14.6 \pm 1.2^{b,d}$	$4.9 \pm 1.2^{a,c}$	$15.3 \pm 2.1^{b,d}$	$14.5 \pm 1.8^{b,d}$	$16.2 \pm 0.9^{b,d}$	$1.6 \pm 0.3^{a,c}$	12.1 ± 1.2^{d}
Kidney	$5.1 \pm 0.7^{b,d}$	$7.7 \pm 0.2^{b,d}$	$2.6 \pm 0.4^{a,c}$	2.6 ± 0.2	$5.3 \pm 0.3^{b,d}$	$6.9 \pm 0.1^{b,d}$	$1.7 \pm 0.3^{a,c}$	3.4 ± 0.01
Abdominal nodes	0.56 ± 0.19	0.23 ± 0.11	1.6 ± 0.2^{a}	0.08 ± 0.03^{b}	0.10 ± 0.05^{b}	0.6 ± 0.3^{b}	0.69 ± 0.47	$0.01 \pm 0.002^{a,b,d}$
Diaphragm	0.81 ± 0.16^{b}	0.82 ± 0.07^{b}	$17.9 \pm 3.0^{a,c,d}$	0.58 ± 0.10^{b}	0.54 ± 0.06^{b}	1.1 ± 0.3^{b}	0.36 ± 0.04^{b}	0.63 ± 0.14^{b}
Pericardium	0.15 ± 0.05	0.22 ± 0.05	n/c	n/c	3.1 ± 1.4	4.0 ± 0.4	10.7 ± 3.5	1.6 ± 0.06
Urine	$36.0 \pm 4.2^{b,d}$	$36.4 \pm 5.3^{b,d}$	18.3 ± 2.7^{a}	$10.0 \pm 1.8^{a,c}$	25.5 ± 1.4^{d}	30.3 ± 2.1^{d}	$10.2 \pm 1.7^{a,c}$	13.0 ± 0.8^{a}
Feces	3.7 ± 0.6	8.0 ± 1.6	3.2 ± 0.8	0.91 ± 0.26	1.7 ± 0.3	3.3 ± 1.6	1.3 ± 0.4	2.4 ± 1.0
Bowel	13.6 ± 1.4	19.3 ± 0.5	13.5 ± 2.9	6.8 ± 0.5	11.5 ± 1.7	19.5 ± 2.3	3.7 ± 0.5	11.6 ± 1.1

n/c, not collected. $p < 0.05$. One way ANOVA. Values are the mean ± SEM.

(0.5): 0.5 mg phospholipid (biotin-liposomes) and 0.5 mg avidin. (5.0): 5.0 mg phospholipid (biotin-liposomes) and 5.0 mg avidin. (0.5)c and (5.0)c: Control groups, saline was injected instead of avidin.

IP-Pleu: ^{99m}Tc-blue-biotin-liposomes injected in peritoneum and avidin injected in pleural space. Pleu-IP: ^{99m}Tc-blue-biotin-liposomes injected in pleural space and avidin injected in peritonuem. For Pleu injections a total volume of either 0.03 or 0.3 mL was injected for each agent. For the IP injections, either biotin-liposomes or avidin were diluted in saline to a total volume of 1.0 mL.

[a]Versus IP/Pleu(0.5).
[b]Versus IP/Pleu(5.0).
[c]Versus Pleu/IP(0.5).
[d]Versus Pleu/IP(5.0).

lower ($p < 0.05$) when the 5.0 mg dose was injected either by IP/Pleu or Pleu/IP method, but no difference was observed between these injection methods. For liver, no difference was observed between any of the groups and the average %ID was 16.9. For spleen, liposome accumulation in groups IP/Pleu(5.0) and Pleu/IP(5.0) was significantly lower (%ID < 5%) from the other groups (%ID > 12%) ($p < 0.05$). Similar situation was observed for kidney, experimental groups injected with 5.0 mg dose showed the lower accumulation of liposomes (%ID < 2.6%) as compared with the experimental groups injected with the 0.5 mg dose (%ID > 5%). For diaphragm, group IP/Pleu(5.0) showed the highest accumulation of liposomes (%ID 17.9%) as compared with all the other groups (average %ID was 0.7) ($p < 0.05$).

From these results, a trend was observed indicating that the 5.0 mg dose, injected either by IP/Pleu or Pleu/IP method, results in the best injected dose, as compared with the 0.5 mg dose in terms of mediastinal node targeting and organ biodistribution.

Table 2 shows the biodistribution results for experimental and control groups at 22 h, in terms of the %ID per gram (%ID/g). These results also show the same trend of higher mediastinal node, diaphragmatic and pericardial lymphatic targeting and lower accumulation of liposomes in other organs when the 5.0 mg dose is injected, using either IP/Pleu or Pleu/IP method. For example, low accumulation of liposomes is observed in spleen ($p < 0.05$) when the 5.0 mg dose is used.

Related to the method of injection, the more significant difference was observed in terms of the organ distribution and lymphatic targeting. High accumulation of liposomes in diaphragm (19.2%/g) with method IP/Pleu(5.0), and in pericardium (86.6%/g) with method Pleu/IP(5.0) was observed. No significant difference was observed in terms of mediastinal node targeting.

Figure 5 shows the ratio of the %ID/g of the ^{99m}Tc-blue-biotin-liposomes in mediastinal nodes and in other major organs for the experimental groups. When method Pleu/IP(5.0) was used, around 7700-fold more liposomes accumulated in mediastinal nodes than in blood; around 80-fold as compared to spleen; and over 200-fold as compared to kidney. For kidney, no difference was observed when the 5.0 mg dose was used either by IP/Pleu or Pleu/IP method. However, a significant difference was observed in liver when comparing method IP/Pleu(5.0) versus Pleu/IP(5.0) (234- vs. 84-fold).

Blue Dye Delivery

Table 3 shows the amount of blue dye delivered per organ and per gram-tissue by liposomes in mediastinal nodes and other organs. The values presented in Table 3 were calculated from the %ID/organ and %ID/g presented in Tables 1 and 2 and the total amount of phospholipid and blue dye measured in the blue-biotin-liposomes. We found that the amount of blue dye encapsulated in liposomes is proportional to the amount of phospholipid injected. In this way, the amount of blue dye injected for the 0.5 mg dose was calculated to be 15 μg and for the 5.0 mg was 150 μg. Results indicated that when larger 5.0 mg dose is injected either by IP/Pleu or Pleu/IP method, mediastinal node accumulation of blue dye is at least 20-fold higher as compared with the 0.5 mg dose. However, accumulation in other important organs was also high.

Comparing injection methods, Pleu/IP(5.0) showed lower deposition of blue dye in blood and spleen than method IP/Pleu(5.0). It is important to mention that when blue-biotin-liposomes are IP injected, high levels of accumulation were observed in diaphragm and the associated lymphatics. When intrapleurally injected, accumulation of blue-biotin-liposomes was observed in pericardium and pleural lymphatics (Fig. 6).

DISCUSSION

There are several diseases involving the lymphatic system during the early stages of disease progression. For example, infections associated with fungal, bacterial, and viral agents are commonly found in lymph nodes.[23–25] In cancer processes, carcinomas, melanomas, and synovial sarcomas metastasize using the lymphatics by invading lymph nodes that often harbor residual tumor cells that can cause tumor recurrence even after the treatment of the primary tumor. For these reasons, an efficient therapy for diseases affecting the lymphatics should rely on the availability of drugs retained by lymph nodes.[2,26,27]

Tumors and infections involving body cavities such as abdominal and thoracic cavities often spread by lymphatic disseminations leading to their residence in regional and distant lymph nodes, such as mediastinal lymph nodes.[4–6,24,28] In the present work, our interest was to show that intracavitary injection (intraperitoneal and intrapleural) of the avidin/biotin-liposome system could

Table 2. Biodistribution Results (%ID/g) 22 h after Injection in Peritoneum and Pleural Space of ^{99m}Tc-Blue-Biotin-Liposomes and Avidin

	IP-Pleu				Pleu-IP			
	(0.5) ($n=5$)	(0.5)c ($n=3$)	(5.0) (n = 4)	(5.0)c ($n=3$)	(0.5) ($n=4$)	(0.5)c ($n=3$)	(5.0) ($n=4$)	(5.0)c ($n=3$)
Organ	%ID/g				%ID/g			
Blood	0.55 ± 0.06^{d}	0.8 ± 0.3^{d}	0.2 ± 0.1^{d}	1.1 ± 0.12^{d}	$0.56 \pm 0.11^{b,d}$	$1.16 \pm 0.22^{b,d}$	$0.024 \pm 0.006^{a,c}$	$0.95 \pm 0.02^{b,d}$
Heart	0.12 ± 0.01	0.25 ± 0.06	0.04 ± 0.02	0.2 ± 0.02	0.1 ± 0.02	0.3 ± 0.01	0.06 ± 0.02	0.2 ± 0.03
Lung	0.31 ± 0.03	0.5 ± 0.1	0.2 ± 0.04	0.58 ± 0.13	0.5 ± 0.07	1.3 ± 0.1	0.35 ± 0.06	0.5 ± 0.07
Thymus	0.4 ± 0.1	0.4 ± 0.05	n/c	n/c	2.0 ± 0.3	3.5 ± 0.8	0.7 ± 0.2	1.3 ± 0.2
Mediastinal nodes	112.1 ± 12.0	$21.0 \pm 2.3^{a,b,d}$	257.5 ± 20.1^{c}	$8.6 \pm 2.7^{a,b,c,d}$	$74.9 \pm 26.5^{b,d}$	$40.0 \pm 9.0^{a,b,d}$	185.8 ± 18.4^{c}	$23.3 \pm 6.4^{a,b,d}$
Liver	1.86 ± 0.24	1.8 ± 0.2	1.1 ± 0.2	0.93 ± 0.14	2.0 ± 0.3	1.8 ± 0.15	2.2 ± 0.4	0.8 ± 0.1
Spleen	$19.5 \pm 2.3^{b,d}$	$24.4 \pm 3.1^{b,d}$	$6.7 \pm 1.3^{a,c,d}$	$29.4 \pm 3.0^{b,d}$	$21.9 \pm 2.5^{b,d}$	$24.8 \pm 1.9^{b,d}$	$2.4 \pm 0.5^{a,b,c}$	$15.5 \pm 1.2^{b,c,d}$
Kidney	$2.5 \pm 0.4^{b,d}$	$3.8 \pm 0.05^{b,d}$	$0.99 \pm 0.06^{a,c}$	1.7 ± 0.1^{d}	$2.4 \pm 0.03^{b,d}$	$3.7 \pm 0.1^{b,d}$	$0.81 \pm 0.15^{a,c}$	1.49 ± 0.05^{d}
Abdominal nodes	28.7 ± 8.9	12.3 ± 4.7	111.0 ± 40.1	7.5 ± 3.5	11.1 ± 6.6	4.4 ± 1.8	39.5 ± 31.8	1.7 ± 0.9
Diaphragm	1.0 ± 0.2^{b}	1.1 ± 0.05^{b}	$19.2 \pm 2.4^{a,c,d}$	0.9 ± 0.1^{b}	0.55 ± 0.06^{b}	$1.6 \pm 0.3^{b,c,d}$	0.4 ± 0.06^{b}	0.8 ± 0.2^{b}
Pericardium	$2.0 \pm 0.6^{c,d}$	$3.4 \pm 0.3^{b,c,d}$	n/c	n/c	$29.3 \pm 9.0^{a,d}$	$68.2 \pm 3.8^{a,c}$	$86.6 \pm 10.6^{a,c}$	$17.2 \pm 1.6^{a,c,d}$
Urine (mL)	0.9 ± 0.13	1.05 ± 0.2^{d}	0.8 ± 0.1	0.24 ± 0.02	1.6 ± 0.36	1.1 ± 0.06^{d}	0.6 ± 0.2	0.32 ± 0.01
Feces	0.2 ± 0.04	0.4 ± 0.06	0.16 ± 0.03	0.03 ± 0.007	0.11 ± 0.03	0.14 ± 0.07	0.07 ± 0.01	0.11 ± 0.04
Bowel	0.3 ± 0.05	0.53 ± 0.05	0.26 ± 0.05	0.15 ± 0.001	0.3 ± 0.04	0.5 ± 0.05	0.85 ± 0.01	0.27 ± 0.02

n/c, not collected. $p < 0.05$. One way ANOVA. Values are the mean ± SEM.

(0.5): 0.5 mg phospholipid (biotin-liposomes) and 0.5 mg avidin. (5.0): 5.0 mg phospholipid (biotin-liposomes) and 5.0 mg avidin. (0.5)c and (5.0)c: Control groups, saline was injected instead of avidin. IP-Pleu: ^{99m}Tc-blue-biotin-liposomes injected in peritoneum and avidin injected in pleural space. Pleu-IP: ^{99m}Tc-blue-biotin-liposomes injected in pleural space and avidin injected in peritonuem. For Pleu injections a total volume of either 0.03 or 0.3 mL was injected for each agent. For the IP injections, either biotin-liposomes or avidin were diluted in saline to complete a total volume of 1.0 mL.

[a]Versus IP/Pleu(0.5).
[b]Versus IP/Pleu(5.0).
[c]Versus Pleu/IP(0.5).
[d]Versus Pleu/IP(5.0).

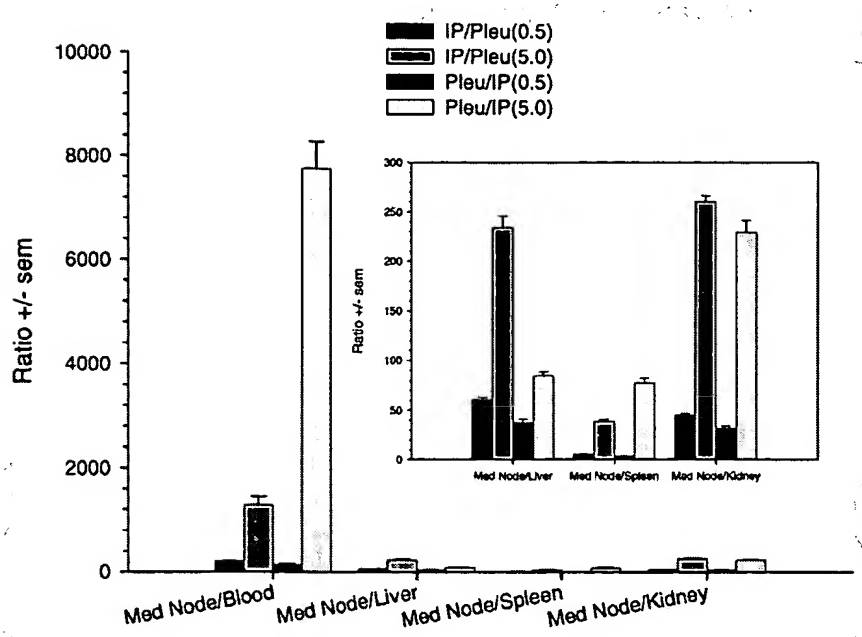

Figure 5. This figure depicts the preferential accumulation of ^{99m}Tc-blue-biotin-liposome aggregate in mediastinal nodes as compared with other major organs. The inset shows a close up of the ratio results for liver, spleen and kidney that are difficult to observe in the large graph. Ratio values were calculated using the %ID/g tissue values from Table 2.

potentially be used to deliver therapeutic and prophylactic drugs to the lymphatics that drain these cavities. Using intracavitary injection methods, we showed that not only mediastinal node targeting can be accomplished, but also targeting of important diaphragmatic and pleural lymphatics can be observed.

The injection technique described in the present work was planned to produce a liposome aggregate mainly in the lymphatic channels that drain the cavities and in mediastinal nodes. The initial hypothesis was that the injection of avidin in peritoneum and biotin-liposomes in pleural space, and vice versa, could produce a more effective retention of liposomes in lymphatics channels and mediastinal nodes avoiding liposome aggregation in the cavity of injection. In previous biodistribution studies,[16,17,29] we observed that when injection of both agents was made in the same cavity, a liposome aggregate still was found trapped in the cavity of injection even after 22 h, regardless of whether the agents were injected at different times. Movement of liposomes from the injection site could be beneficial during adjuvant therapeutic treatments in terms of reducing toxic side effects of the therapeutic drug transported by liposomes and increasing accumulation of the therapeutic agent in the target site.

The results presented in this work indicated that the method of intracavitary injection used (IP/Pleu or Pleu/IP) does not play an important role in terms of mediastinal node targeting. The importance of the method of injection was more significant in terms of the lymphatic distribution and pharmacokinetics of the liposomes (see "Results").

It was found that when the avidin/biotin-liposome system was intracavitary injected as described in the present work, the most relevant factor affecting the ultimate targeting of mediastinal lymph nodes was the dose injected (Fig. 4A). When the IP/Pleu(5.0) method was used, high levels of ^{99m}Tc-blue-biotin-liposomes were observed and detected in the diaphragm, the collecting lymphatics in the pleura that cover the diaphragm, the parasternal lymphatic channels in the sternum and mediastinal nodes (Tabs. 1 and 2; Figs. 3D and 6). When the IP/Pleu(0.5) method was used, liposomes moved quickly into blood circulation and spleen (Figs. 2 and 3C), and retention of liposomes in the lymphatics and mediastinal nodes was much lower (Tabs. 1 and 2).

These results have significant value in terms of targeting prophylactic drugs to the lymphatics that drain the abdominal cavity and could be useful in the prevention of dissemination of peritoneal diseases. As previously described, the

Table 3. Total Amount of Blue Dye Delivered by Liposomes in Mediastinal Nodes and other Important Organs

Organ	IP/Pleu (0.5) ($n=5$)	IP/Pleu (5.0) ($n=4$)	Pleu/IP (0.5) ($n=4$)	Pleu/IP (5.0) ($n=4$)	IP/Pleu (0.5) ($n=5$)	IP/Pleu (5.0) ($n=4$)	Pleu/IP (0.5) ($n=4$)	Pleu/IP (5.0) ($n=4$)
	Dose (μg)/Organ[a]					Dose (μg)/g-tissue[a]		
Mediastinal Nodes	0.7 ± 0.1	15.0 ± 1.5	0.3 ± 0.04	11.9 ± 0.75	16.7 ± 1.8	384.4 ± 36.1	11.2 ± 4.0	277.4 ± 27.5
Blood	1.4 ± 0.2	6.0 ± 3.0	1.6 ± 0.3	0.7 ± 0.1	0.08 ± 0.009	0.3 ± 0.15	0.08 ± 0.02	0.04 ± 0.01
Liver	3.0 ± 0.3	22.0 ± 5.2	3.1 ± 0.2	34.2 ± 7.0	0.28 ± 0.036	1.64 ± 0.30	0.3 ± 0.04	3.3 ± 0.6
Spleen	2.0 ± 0.3	7.3 ± 1.8	2.2 ± 0.3	2.4 ± 0.4	2.9 ± 0.34	10.0 ± 1.9	3.3 ± 0.4	3.6 ± 0.7
Kidney	0.8 ± 0.1	3.9 ± 0.6	0.8 ± 0.04	2.5 ± 0.4	0.37 ± 0.06	1.48 ± 0.09	0.36 ± 0.004	1.2 ± 0.2
Pericardium	0.2 ± 0.01	n/c	0.5 ± 0.2	16.0 ± 5.2	0.3 ± 0.09	n/c	4.4 ± 1.3	129.3 ± 15.8
Diaphragm	0.1 ± 0.02	26.7 ± 4.5	0.1 ± 0.01	0.5 ± 0.1	0.15 ± 0.03	28.7 ± 3.6	0.08 ± 0.01	0.6 ± 0.9

n/c, not collected. Values are the mean ± SEM.
Total blue dye dose injected: IP/Pleu(0.5) and Pleu/IP(0.5): 15 μg; IP/Pleu(5.0) and Pleu/IP(0.5): 150 μg.
[a]Based on the %ID/Organ and %ID/g values presented in Tables 1 and 2.

lymphatics in the diaphragm play an important role in the dissemination of tumor cells, and pathogens from the peritoneal cavity.[6] The importance of diaphragm targeting for the treatment and imaging of lung cancer metastasis[30] and for animal model studies of Duchenne muscular dystrophy has also been reported.[31]

In terms of the physical and chemical factors that affect the removal of liposomes from the abdominal cavity, Sadzuka et al.[32] described the role of lipid composition, charge, size, and liposome surface modification, but no mention was made related with the lipid dose injected. An important conclusion from that study was that large liposomes (>400 nm) are more effective against peritoneal dissemination than small liposomes (150 nm). A similar conclusion was also reported by Hirano and Hunt.[33] In this study, liposome retention in mediastinal lymph nodes was better with large liposomes than small liposomes. However, they also reported that stability of liposomes as drug carriers is reduced with increasing liposome size. Small liposomes (<200 nm) are more stable as drug carriers because they reduce the possibility of leakage of therapeutic radionuclides or chemotherapeutics encapsulated inside of the liposomes and thus improve the efficacy of the treatment by reducing toxic side effects. In the present work, we have shown that small stable biotin-liposomes (<150 nm) when administered with avidin using the IP/Pleu(5.0) method, can be used for the targeting of the lymphatics that drain the abdominal cavity, resulting in high levels of liposome accumulation in diaphragmatic lymphatic channels and mediastinal nodes.

Similar behavior related with the injection dose was observed when ^{99m}Tc-blue-biotin-liposomes were injected into the pleural space and avidin was injected in peritoneum (Pleu/IP(5.0) method). Even though it is difficult to differentiate the activity of ^{99m}Tc-blue-biotin-liposomes in the thoracic lymphatics and in mediastinal nodes on the images in the early hours after injection, it was evident that the large dose (5.0 mg) resulted in high retention (~10% ID) of liposomes in mediastinal nodes after 12 and 22 h (Fig. 4A and Tab. 1). It was observed that using the 0.5 mg dose, the aggregation of liposomes is low and as a consequence, retention in lymphatic channels and mediastinal nodes is minimal. During biodistribution studies, following injection of liposomes in the pleural space, high levels of injected dose were detected in pericardium, mediastinal nodes and

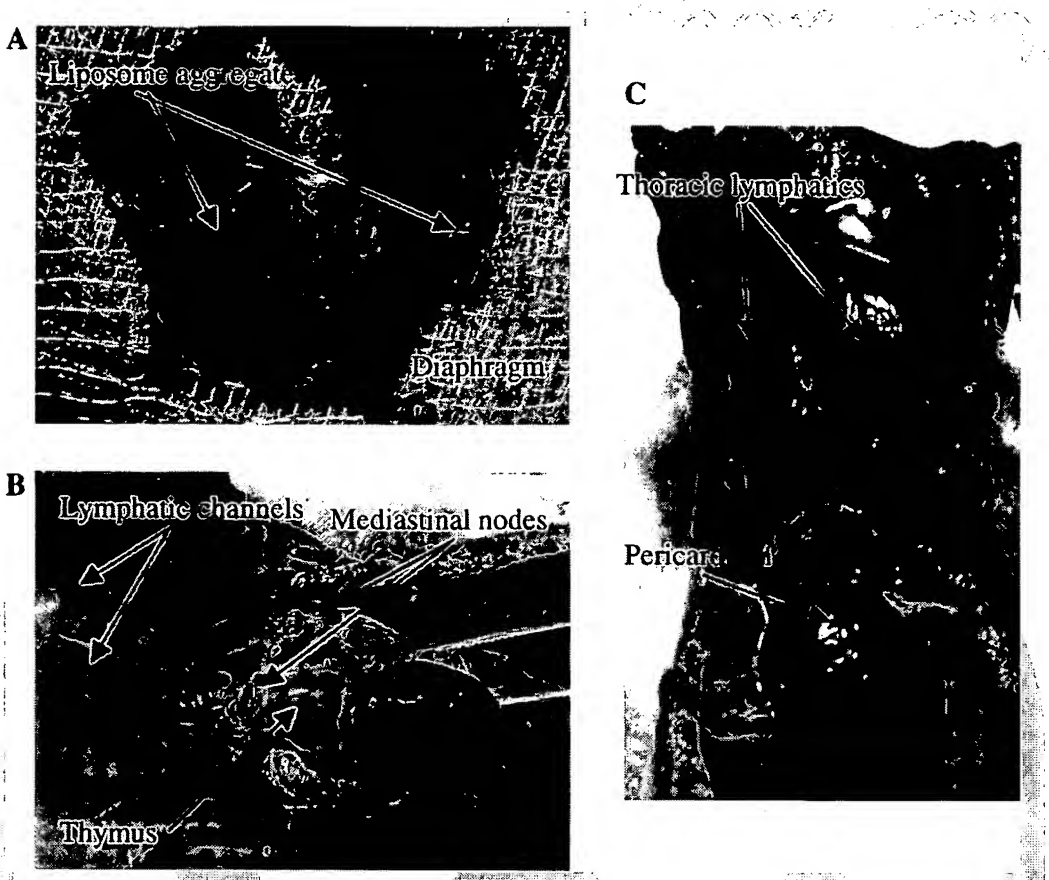

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Figure 6. (A) Photograph of the diaphragm and the pleural lymphatics that cover the upper part of the diaphragm showing the accumulation of blue-liposome aggregate; (B) photograph of the mediastinal nodes and the parasternal lymphatics channels that drain the upper part of the diaphragm into mediastinal nodes; (C) Photograph showing the accumulation of blue-liposome aggregate in the pericardial lymphatics and the thoracic lymphatics at the chest wall that drain pleural space.

the chest wall (Fig. 6C) when the 5.0 mg dose was used, but accumulation in diaphragm and pleural space was minimal (Tabs. 1 and 2). It is important to mention that the tissue collected as pericardium also includes the connective tissue that connects the pericardium with diaphragm, and some of the collecting lymphatic channels that cover the pleural part of the diaphragm. It is not totally clear the pathways in which liposomes are cleared from pleural space,[34] but it was evident that at some point, the biotin-liposomes join the avidin in some of the lymphatic channels that drain into mediastinal nodes.

The potential use of liposome encapsulated anticancer drugs as a method to reduce the frequency and growth rate of tumor metastasis in lymph nodes has been described previously.[35,36] In these studies, the authors reported that the use of *cis*-diamminedichloroplatinum(II) (dose: 0.4-0.6 mg/kg-body), hydrocortisone (10 mg/kg-body) and melphalan (0.125 mg/kg-body) encapsulated in liposomes results in an effective procedure for the prophylactic treatment of tumor metastasis and growth in lymph nodes after IP injection. To exemplify the delivery of drugs carried by liposomes in mediastinal nodes and other organs and tissues, in the present work we injected a total dose of 0.15 mg (5.0 mg/kg-body wt) of blue dye encapsulated in liposomes. The results presented in Table 3 showed that a total dose of 0.015 mg was delivered to the mediastinal nodes (384 μg/g-tissue) and ~0.03 mg in diaphragm (~diaphr-diaphragmatic lymphatics) (~30 μg/g-tissue) when liposomes were injected IP/Pleu(5.0). With Pleu/IP(5.0) method, a dose of 0.012 mg (277 μg/g-tissue) in mediastinal nodes and 0.016 mg (129 μg/g-tissue) in pericardium (~pleural lymphatics) was delivered. These results showed the potential

of the avidin/biotin-liposome system for the direct delivery of prophylactic drugs into mediastinal lymph nodes.

One problem observed during these experiments was the high levels (at least twofold) of liposome accumulation detected in liver as compared with the results observed in previous experiments where the same dose (5.0 mg) of avidin and biotin-liposomes were injected in the same cavity.[16,17] One possible explanation is related to the rate of removal of the agents in each cavity. We have already observed that fluid removal from peritoneum is much faster than the removal of fluid from pleural space. It is possible that this faster drainage of either avidin or blue-biotin-liposomes from the peritoneum will reduce the rate of liposome aggregation, giving time for the biotin-liposomes to gain quick access to the blood circulation and then collect in the liver. Other possibilities for improving the injection method in terms of injection sequence, time between injections and number of avidin injections should be pursued in future studies to discover methods to further reduce the rate of liposome access to the circulation.

Future studies should be performed in abnormal animals affected by diseases that spread using the lymphatic system. The results presented in this work could be used as a reference for assessing changes in the pattern of lymphatic absorption and mediastinal node targeting from a cavity affected by an advanced disease process. As previously reported, the lymphatic drainage patterns could change under the presence of cancer or infection.[37]

The targeting of the mediastinal nodes and lymphatics that drain peritoneal and pleural space could help in the treatment and control of infection spread from both cavities. In the case of tumor spread, the targeting of prophylactic drugs into mediastinal nodes could be potentially used to control tumor metastasis and tumor cell seeding in the nodes. The reduction of liposome accumulation in the cavity of injection using this injection technique could improve the targeting of the lymphatics and also reduce potential toxic side effects of the encapsulated drug transported by the liposomes.

It is important to mention that the clinical use of avidin and streptavidin-biotin systems for targeting or pretargeting strategies may be limited, due to the immunogenicity of avidin and streptavidin. However, this problem of immunogenicity has been investigated using recombinant, biochemically modified, and PEG conjugation.[38,39] Several authors have reported a significant reduction in immunogenicity of many proteins that have been covalently linked with PEG.[40,41] Although some reduction in the activity of proteins was observed,[42,43] Marshall et al. have reported a galactosylated form of streptavidin (gal-streptavidin) conjugated with PEG that has shown a significant reduction in its immunogenicity while still allowing the binding of biotinylated antibodies.[44] The use of these PEG-avidin and streptavidin bioconjugates could solve the possible limited application of the biotin-liposomes for targeting of lymph nodes.

The results presented in this work using intracavitary injection of the avidin/biotin-liposome system suggest that further studies of the intracavitary injection of this system should be pursued.

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